



***In vitro* Callus Selection in *Brassica* species**

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## Abstract

The study mainly concerns the potential of *in vitro* selection as an aid to plant breeders. Three species, of the genus *Brassica*, were used in the investigations as model crop plants. Callus from these species were selected for NaCl tolerance. The study has three main sections dealing with the needs of tissue culture in *Brassica* species, *in vitro* selection of callus for NaCl tolerance and estimation of somaclonal variation.

The first section evaluates the potential of different *Brassica* species and explant sources for *in vitro* culture. The study also describes investigations in the optimum growth regulator concentrations for continued callus cultures and subsequent plant regeneration, in these *Brassica* species. All the explant types i.e. leaf discs with vein, leaf discs without vein, petioles and hypocotyls, of all the species studied, showed successful callus induction and subsequent callus cultures, but with varying frequencies. Plant regeneration from callus was not successful in all the species and with all the explant sources. Curly kale (*Brassica oleracea* L.) showed the most successful plant regeneration. Among the explant sources, hypocotyls demonstrated the most potential with respect to plant regeneration. Optimum concentrations of NAA and BAP were different for different species. In general, BAP and NAA in balance produced more callus; and as expected, BAP enhanced shoot formation while NAA promoted root formations.

The second section deals with procedures and problems associated with the *in vitro* selection of different *Brassica* species for salt tolerance. In addition the study also deals with the maintenance of selected callus and stability of the selected calli, with respect to salt tolerance. Nevertheless, callus was selected successfully from all the *Brassica* species, studied. In general, selection was found effective giving more salt tolerant cell lines from selected callus in comparison with the unselected callus. However, the stability of the salt tolerance varied between the species. Only *Brassica rapa* L. callus showed stable salt tolerance even after sub-culturing on salt free medium after selection, for few generations. The other two species showed unstable salt tolerance. In these species the selected callus was not significantly more salt tolerant than unselected callus, when exposed to the salt free medium after selection.

The third section deals with the an estimation of the variation found with regenerated plants and callus from different culture cycles and culture conditions. Somaclonal variation was detected and quantified using RAPD-PCR analysis. Regenerated plants and callus from different cultures, showed different frequencies of variation to the parent plants. In general, variation showed by different cultures ranged from 1 to 11%. No DNA polymorphism was observed specific to the NaCl tolerance. Effect of culture duration on somaclonal variation was also not detected by RAPDs analysis.

The final discussion reviews *in vitro* selection of callus for salt tolerance. The wider applicability of *in vitro* selection for crop improvement along with limitations and future prospects are also reviewed in the context of somaclonal variation and its application.

## DECLARATION

*This thesis has been composed by me and is based on the results of investigation carried out by myself. The thesis has not been accepted for any application for a degree. All information and assistance obtained from other sources has been specifically acknowledged by means of reference.*

SHAFIQ AHMAD

January 1996

***Dedication to My***

***Son  
Sadi  
and  
Daughter  
Sundas***



**“The only clue what man can do is what man has done”**

(R.G. Collingwood, *The Idea of History*, Oxford, 1946)

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# Contents

<b>List of Tables</b> .....	XIII
<b>List of Figures</b> .....	XV
<b>List of Abbreviations</b> .....	XVIII
<b>1. INTRODUCTION</b> .....	1
<b>1.1. General introduction</b> .....	1
<b>1.2. Tissue culture</b> .....	4
<b>1.3. Somaclonal variation</b> .....	7
1.3.1. Nature of variation.....	7
1.3.2. Frequency of occurrence .....	8
1.3.3. Genetic basis of somaclonal variation .....	9
1.3.4. Somaclonal variation in <i>Brassica</i> species.....	9
1.3.5. Somaclonal variation for salt tolerance .....	10
1.3.6. General uses.....	11
1.3.7. Detection of somaclonal variation.....	12
<b>1.4. <i>In vitro</i> selection</b> .....	14
1.4.1. Selection strategies.....	15
1.4.1.1. Resistance selection.....	15
1.4.1.2 Visual selection.....	16
1.4.1.3. Counterselection.....	16
1.4.1.4. Total selection.....	16
1.4.2. Choice of material .....	17
1.4.2.1. Callus cultures.....	17
1.4.2.2. Cell suspension cultures .....	18
1.4.2.3. Plated cells.....	19
1.4.2.4. Protoplast cultures .....	20
1.4.3. <i>In vitro</i> selection for salt tolerance.....	20
<b>1.5. Aims and objectives</b> .....	22

<b>2. MATERIALS METHODS</b> .....	24
2.1. General conditions .....	24
2.2. Plant material.....	24
2.3. Preparation of culture media.....	25
2.4. Explant sources and their preparation.....	25
2.4.1. Surface sterilisation and sowing of seed.....	26
2.4.2. Sterilisation of leaves and petioles .....	28
2.4.3. Preparation of explants.....	28
2.5. Callus initiation.....	29
2.6. Sub-culture and maintenance.....	29
2.7. Assessment of callus growth .....	30
2.8. <i>In vitro</i> selection, maintenance and stability tests.....	31
2.9. <i>In vitro</i> regeneration .....	32
2.10. Hardening and transfer of plants .....	32
2.11. Bio-molecular analysis .....	33
2.11.1. DNA isolation.....	33
2.11.2. DNA visualisation .....	34
2.11.3. Determination of amount and purity of DNA.....	35
2.11.4. DNA amplifications.....	35
2.11.5. Genetic similarity estimate.....	36
 <b>3. BASIC TECHNIQUES FOR TISSUE CULTURES OF <i>BRASSICA</i> SPECIES</b> .....	 37
3.1. Introduction .....	37
3.2. Selection of explant type suitable for <i>in vitro</i> culture and regeneration .....	39
3.2.1. Experimental procedure .....	39
3.2.2. Callus initiation .....	40

3.2.3. Shoot regeneration.....	43
<b>3.3. Optimisation of NAA and BAP for callus production.....</b>	<b>47</b>
3.3.1. Experimental procedure .....	47
3.3.2. Cultures of curly kale .....	48
3.3.2.1. Effect of NAA and BAP on callus induction from hypocotyl explants of curly kale (a).....	48
3.3.2.2. Effect of NAA and BAP on callus induction from hypocotyl explants of curly kale (b).....	49
3.3.2.3. Effect of NAA and BAP on callus maintenance of curly kale. ....	51
3.3.3. Culture of winter oilseed rape.....	52
3.3.3.1. Effect of NAA and BAP on callus induction from hypocotyl explants of winter oilseed rape (a).....	52
3.3.3.2. Effect of NAA and BAP on callus induction from hypocotyl explants of winter oilseed rape (b).....	52
3.3.3.3. Effect of NAA and BAP on callus maintenance of winter oilseed rape.....	53
3.3.4. Culture of <i>Brassica rapa</i> (R.C.) .....	55
3.3.4.1. Effect of NAA and BAP on callus induction from hypocotyl explants of <i>Brassica rapa</i> (a).....	55
3.3.4.2. Effect of NAA and BAP on callus induction from hypocotyl explants of <i>Brassica rapa</i> (b).....	55
3.3.4.3. Effect of NAA and BAP on callus maintenance of <i>Brassica rapa</i> . ....	56
<b>3.4. Optimisation of NAA and BAP for shoot regeneration.....</b>	<b>58</b>
3.4.1. Experimental procedure .....	59
3.4.2. Shoot regeneration in curly kale .....	60
3.4.3. Shoot regeneration in winter oilseed rape .....	60
3.4.4. Shoot regeneration in <i>Brassica rapa</i> (R.C.).....	61
<b>3.5. Root formation on the regenerated shoots .....</b>	<b>62</b>
3.5.1. Procedure .....	62
3.5.2. Results.....	62
<b>3.6. Discussion.....</b>	<b>64</b>

<b>4. IN VITRO CALLUS SELECTION FOR SALT TOLERANCE</b> .....	73
<b>4.1. Introduction</b> .....	73
<b>4.2. Dose response curve of NaCl for different cultures in <i>Brassica</i> species</b> .....	75
4.2.1. Determination of selective concentrations of NaCl for <i>in vitro</i> callus selection.....	76
4.2.1.1. Experimental procedure .....	76
4.2.1.2. Results.....	77
4.2.2. Effect of different concentration of NaCl on callus induction, using hypocotyl segments of different <i>Brassica</i> species, as explant source.....	81
4.2.2.1. Experimental procedure. ....	81
4.2.2.2. Results.....	81
4.2.3. Effect of NaCl on seed germination and seedling growth of different <i>Brassica</i> species.....	85
4.2.3.1. Experimental procedure .....	85
4.2.3.2. Results.....	85
<b>4.3. <i>In vitro</i> selection for salt tolerance</b> .....	90
4.3.1. Experimental procedure .....	90
4.3.2. Selection of curly kale callus for 1% NaCl resistance.....	91
4.3.3. Selection of winter oilseed rape callus, tolerant to 0.75% NaCl. ....	92
4.3.4. Selection of <i>Brassica rapa</i> callus tolerant to 1% NaCl.....	92
<b>4.4. Stability for salt tolerance</b> .....	94
4.4.1. Experimental procedure .....	95
4.4.2. Stability tests for curly kale callus.....	97
4.4.3. Stability tests for winter oilseed rape .....	102
4.4.4. Stability tests for <i>Brassica rapa</i> callus.....	104
<b>4.5. Discussion</b> .....	109

<b>5. SOMACLONAL VARIATION .....</b>	<b>115</b>
<b>5.1. Introduction .....</b>	<b>115</b>
<b>5.2. DNA extraction .....</b>	<b>117</b>
<b>5.3. RAPD-PCR analysis .....</b>	<b>119</b>
5.3.1. Estimation of MgCl <sub>2</sub> quantity for PCR reactions .....	119
5.3.2. Study for primers .....	120
5.3.3. Variations in cultures .....	122
5.3.4. RAPD profile by the primer OPA-06 .....	126
5.3.5. RAPD profile by the primer OPA-07 .....	126
5.3.6. RAPD profile by the primer OPD-09 .....	130
5.3.7. RAPD profile by the primer OPD-10 .....	130
5.3.8. RAPD profile by the primer OPD-13 .....	133
5.3.9. Presence of chimera in tissue culture .....	135
5.3.10. Genetic differences between selected and un-selected cultured material.....	136
5.3.11. Effect of culture duration on somaclonal variation. ....	136
<b>5.4. Discussion.....</b>	<b>38</b>
 <b>6. GENERAL DISCUSSIONS AND POSSIBILITIES FOR FUTURE ESEARCH.....</b>	 <b>144</b>
 <b>7. REFERENCE CITED .....</b>	 <b>149</b>
 <b>8. APPENDICES.....</b>	 <b>175</b>



## List of Tables

Table 2.1. Composition of MS-medium used in tissue culture.....	27
Table 3.1. Combinations of NAA/BAP added to tissue culture media to study the effect of growth regulators on <i>in vitro</i> cultures.....	40
Table 3.2. Callus induction on different explant sources.....	41
Table 3.3. Results of different $\chi^2$ -tests for callus induction from four different explant sources of 3 <i>Brassica</i> species.....	42
Table 3.4. Shoot initiation on different explant sources.....	43
Table 3.5. Results of different $\chi^2$ -tests for direct shoot regeneration from 4 different explant sources of 3 <i>Brassica</i> species,.....	44
Table 3.6. Combination of NAA/BAP added to the tissue culture media to study the effect of growth regulators on <i>in vitro</i> cultures.....	51
Table 3.7. Combination of NAA/BAP added to the tissue culture media to study the effect of growth regulators on <i>in vitro</i> cultures.....	53
Table 3.8. Combination of NAA/BAP added to the tissue culture media to study the effect of growth regulators on shoot regeneration from the callus of different <i>Brassica</i> species.....	58
Table 3.9. Results of different $\chi^2$ -tests for shoot regeneration on different concentrations of NAA in combination with BAP.....	59
Table 3.10. Explant sources used successfully in different <i>in vitro</i> studies involving <i>Brassica</i> species.....	65

Table 3.11. Successful use of hypocotyl, as explant source for different <i>in vitro</i> culture studies in genus <i>Brassica</i> .....	67
Table 4.1. Results of different t-test for callus growths of different <i>Brassica</i> species on selective and non- selective culture medium. ....	91
Table 5.1. Base sequences and molecular weight of different decamer oligonucleotides from kit 'A' and 'D' of Operon Technologies Inc. ....	122
Table 5.2. Variation in different <i>in vitro</i> cultures of curly kale, estimated by RAPD-PCR amplification.....	124

## List of Figures

Fig. 3.1. Influence of explant type on shoot differentiation frequency from different species of <i>Brassica</i> .....	45
Fig. 3.2. Influence of genotypes and explant type on shoot differentiation frequency. ....	46
Fig. 3.3. Influence of different levels of NAA and BAP on callus induction and subsequent callus culture of Curly Kale.....	50
Fig. 3.4. Effect of different concentrations of NAA and BAP on callus culture of winter oilseed rape. ....	54
Fig. 3.5. Influence of different levels of NAA and BAP on callus induction and subsequent callus culture of <i>Brassica rapa</i> .....	57
Fig. 3.6. Shoot regeneration frequency on different levels of NAA in combination with BAP, in Curly kale.....	60
Fig. 4.1(A). Effect of NaCl levels, on callus cultures of different <i>Brassica</i> species.....	79
Fig 4.1(B). Dose response curve (regression analysis) of callus growth from different <i>Brassica</i> species for different levels of NaCl .....	80
Fig. 4.2. Effect of NaCl levels on callus induction from hypocotyl explants of different <i>Brassica</i> species. ....	84
Fig. 4.3. Effect of various levels of NaCl on seedling fresh weight from different species of <i>Brassica</i> .....	86
Fig 4.4. Difference in osmotic potential of two types of NaCl solutions i.e. with and without MS-medium used in experiments.....	89
Fig. 4.5. <i>In vitro</i> selection of callus of different species of <i>Brassica</i> . ....	93

Fig. 4.6. Salt tolerance of different types of selected and un-selected callus of Curly kale in 9th and 14th culture cycle.....	98
Fig. 4.7. Salt tolerance of regenerants from selected and un-selected callus of Curly kale.....	101
Fig. 4.8. Salt tolerance of different type of selected and un-selected callus of Winter oilseed rape in 9th and 14th culture cycles.....	103
Fig. 4.9. Salt tolerance of different types of selected and un-selected callus of <i>Brassica rapa</i> in 10th and 14th culture cycles.....	107
Fig. 4.10. Salt tolerance of different types of selected and un-selected callus of <i>Brassica rapa</i> in 16th and 17th culture cycle.....	108
Fig. 5.1. DNA extraction through Puregene™ DNA isolation kit. ....	118
Fig. 5.2 Effect of different concentrations of MgCl <sub>2</sub> on PCR-amplifications. ....	118
Fig. 5.3. DNA profiling using different arbitrary oligonucleotides of kit A. ....	121
Fig. 5.4. DNA profiling using different arbitrary oligonucleotides of kit D. ....	121
Fig. 5.5. RAPD profiles of 9 DNA samples generated by the primer OPA-02.....	127
Fig. 5.6. RAPD profiles of 9 DNA samples generated by the primer OPA-06.....	127
Fig. 5.7. RAPD profiles of 38 DNA samples generated by the primer OPA-07.....	128
Fig. 5.8. RAPD profiles of 13 DNA samples generated by the primer OPA-07.....	129

Fig. 5.9. RAPD profiles of 29 DNA samples generated by the primer OPA-09. ....	129
Fig. 5.10. RAPD profiles different DNA samples generated by the primer OPD-09. ....	131
Fig. 5.11. RAPD profiles of 9 DNA samples generated by the primer OPD-10. ....	132
Fig. 5.12. RAPD profiles of 9 DNA samples generated by the primer OPD-13. ....	134
Fig. 5.13. RAPD profiles of 29 DNA samples generated by the primer OPD-13. ....	134
Fig. 5.14. Diagrammatic description of limitations of RAPDs. ....	142

## List of Abbreviations

AFLP	Amplification Fragment Length Polymorphisms
BP/bp	Base pairs (Size of DNA segment)
BAP	6-Benzyl Amino Purine
C	Culture Cycle
CRD	Completely Randomised Design
2,4-D	2,4-Dichlorophenoxyacetic Acid
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate (Adenine, Guanine, Cytocine, Thymine)
GA	Gibberellic Acid
IAA	Indol-3-Acetic Acid
IBA	Indol-3-Butyric Acid
KIN	Kinetin
LSD	Least Significant Differences
M	Maintenance Cycle (after selection, on salt free medium)
Mg	Magnesium or MgCl <sub>2</sub> .
MS-Media	Murashige and Skoog (1962) Basal Media.
NAA	Naphthaleneacetic Acid
OD	Optical Density
<i>P</i>	Probability
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNAs
RFLP	Restriction Fragment Length Polymorphisms
S	Selection Cycle
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error for Mean
TBE	Tris-HCL, Borate EDTA(Ethylenediaminetetraacetic Acid)

# 1. INTRODUCTION

## 1.1. General introduction

The economy of Pakistan is based on agriculture directly or indirectly. In the last few years much progress has been made in agricultural research. Although a number of high yielding, fertiliser-responsive crop varieties, produced through conventional breeding programme have been released, the country is facing many problems in sustaining crop production levels. These problems include: soil salinity, water logging, weeds, insect pests, diseases, high summer temperature and drought. Therefore there is a continuous need to develop new crop varieties which can be successfully grown under these stress conditions.

Selection is well recognised as the most important facet of crop improvement but the efficiency of selection varies substantially with different breeding approaches. Crop improvement through conventional breeding can continue to be effective provided sufficient labour, area for research, favourable and well defined environmental and soil conditions can be assured. Conventional breeding programmes also require an initial variable population and/or land races and a supply of good quality seed in sufficient quantity. The success of any crop improvement programme depends on the exploitation of the genetic variability in the base population. However, for some agricultural crops, there is a lack of genetic variability, which impedes improvement through conventional plant breeding systems. The most readily identifiable weaknesses of conventional plant breeding are that the process take too long and as a result, can be thought of as unresponsive to needs; too uncertain of outcome and too costly. Due to these and many other limitations these approaches have met with variable success when used to improve the tolerance of crops to stress conditions, and consequently an alternative system to conventional crop improvement would appear to be necessary. If *in vitro* culture methods are to contribute to the improvement of plant breeding procedures, they must go at least some way towards correcting one or more of these limitations.

Novel *in vitro* approaches to selection have the theoretical advantage over conventional breeding of creating a large number of potential propagules in highly defined and uniform environments. Studies exploiting *in vitro* culture would seem to indicate that this approach allows speed of selection, combined with high selection

efficiency. Successful selection requires genetic variability; as yet still there is some uncertainty on the source of the variability exploited in *in vitro* selection; but evidence has accumulated suggesting that frequent genetic modifications occur during tissue culture. Many of these modifications manifest themselves as heritable mutations in the progeny of plants regenerated from tissue culture (Scowcroft and Ryan, 1986). Already, plants showing resistance to phytotoxins (Gengenbach *et al.*, 1977), herbicides (Challef and Ray, 1984) and salts (Nabors *et al.*, 1980; Bajaj and Gupta, 1987), that are high yielding (Ogura *et al.*, 1988) have been obtained, and are being incorporated into crop improvement programmes of important agricultural crops such as wheat, rice, maize, potato, sugarcane, brassicas. Some of these have yielded positive results to the extent of new cultivars being released (Bajaj, 1990).

The general aim of this study is to evaluate the potential of *in vitro* callus selection for an abiotic stress. Given the limitation in terms of time available for investigation a species group (*Brassica*) with considerable background prehistory of behaviour in tissue culture, and abiotic stress (salt) of considerable importance were chosen as the component of model system to investigate the problems and limitations of *in vitro* selection.

The reasons for using this species group as research tools, are that a considerable progress has been made in recent years in manipulating *Brassica* spp. *in vitro*. In addition fast growing Rapid Cycling *Brassica* (RC) has emerged as model plant species for research. At present Rapid Cycling *Brassica* serves as model for research in genetics, molecular biology, cell biology, plant biochemistry, population biology and plant breeding (Williams and Hill, 1986). Furthermore brassicas are a diverse group of crop plants with great economic value worldwide and different species of *Brassica* can be successfully grown in different regions of the world.

In other studies, utilising *in vitro* selection, many different approaches have been developed including selection from callus culture, cell line selection, selection from cell plating, anther culture selection, microspore selection and selection from protoplast cultures. For this study it has been decided that *in vitro* selection from callus cultures will be investigated for its practicability and effectiveness.

This introduction is intended to highlight different selection strategies and the utilisation of *in vitro* selection for crop improvement. The genetic basis and the nature of somaclonal variation, its usefulness in *in vitro* selection, appropriate tissue



culture techniques for both plant cell culture and regeneration will be discussed in this review.

## 1.2. Tissue culture

Plant tissue culture is commonly defined as the culture of all types of plant cells, tissue and organs under aseptic conditions (Smith and Drew 1990), although strictly it refers to the culture of undifferentiated mass of plant cells only (Duncan and Widholm, 1986; Street, 1977; George and Sherington, 1984). Plant tissue culture techniques have become a powerful tool for studying many basic and applied problems in plant biology. *In vitro* techniques have many advantages over traditional research methods involving the whole plants, some are mentioned here:

- Only a small amount of space is required to maintain or multiply large number of plants.
- Propagation is carried out in aseptic conditions. Once the culture has been initiated, plants finally produced will be free of bacteria and fungi
- Methods are also available to produce virus-free plants.
- It may be possible to produce the clones of some kinds of plants that are otherwise, very difficult or impossible to propagate vegetatively such as wheat, rice, maize.
- Production can be continued all the year round and is independent of season.
- Cultures can be grown in specific controlled environmental and nutritional conditions required.
- Requires less labour and saves time.

The chief disadvantages of *in vitro* methods are that advanced skills and technologies are required for successful operation. Specific methods may be necessary to obtain optimum results from each species, and in the beginning to establish a laboratory and to start the work it may be expensive.

There are four areas in which application of plant tissue culture are possible, either presently or in the near future:

1. Production of pharmaceuticals and other natural products;
2. The genetic improvement of crops;
3. The recovery of disease-free clones and preservation of valuable germplasm;  
and
4. Rapid clonal multiplication of selected varieties.

Plant cell culture has often been hailed as one of the more significant potential adjuncts to plant improvement. Conventional breeding technology has been

successfully used since the beginning of genetic research, if not before. But due to some limitations regarding time and uncertainty of outcome, new techniques are needed. Plant tissue culture provides a powerful technique to assist a plant breeder in improving the propagation and performance of agriculture, horticulture and forest species (Collin and Dix, 1990). The advantage of *in vitro* systems over conventional breeding procedures for obtaining novel plants lies in the fact that a large number of individuals and potentially different cells can be screened for resistance or tolerance with respect to a particular character in a rapid, space saving and controlled manner under defined conditions (Strauss *et al.*, 1980). This is usually seen in terms of the ability to apply cellular selection for recovering useful genetic variants.

The basic procedure and methods involved in plant tissue culture have been discussed in detail by many research workers (Bhojwani and Razdan, 1983; Mantell *et al.*, 1985; Pierik, 1987). Sterilised explants or *in vitro* cultured single cells or callus are aseptically transferred to a suitable nutrition media. These cultures are incubated in a specific environmental conditions, for a specific period of time and maintained by subsequent sub-culturing. Success in the technology and application of *in vitro* methods is due largely to a better understanding of nutritional requirements of cultured cells (Murashige, 1974; Gamborg *et al.*, 1976; Street, 1977). Murashige and Skoog (1962) developed a revised medium with each element provided in sufficient quantity to ensure that no increase in yield (fresh weight of callus) would result from the introduction of additional amounts in the range ordinarily to be expected in plant tissue extract. This medium, commonly called Murashige-Skoog (MS) medium, is now widely used for general plant tissue culture but especially for morphogenesis, meristem culture and plant regeneration (Murashige, 1974). This is one of the most commonly used tissue culture media and has been used successfully for the *Brassica* species by many researchers (Singh *et al.*, 1991; Shahzadi *et al.*, 1992; Martin *et al.*, 1993)

Besides the nutritional requirement of culture media, *in vitro* culture is greatly influenced by the physical state of the medium as well. In most instances the choice between a liquid or solid medium has been made rather arbitrarily, the decision resting on the available facilities and the accustomed practice of the investigator. With some plant species, the success or failure of tissue culture may depend on whether a liquid or an agar nutrient medium was employed (Murashige, 1974). Callus culture on solid medium is much easier to handle and more successful, especially for regeneration, as compared with culture in a liquid medium. On the other hand, selection in suspension

culture is thought to be more efficient and reliable than on solid medium. This is thought to reflect that not all the cells in a callus piece are uniformly exposed to selective agent and this can result in stress avoidance due to cross-feeding between the cells in close contact with each other (Meredith, 1984). But the regeneration potential of cell lines produced in long term liquid culture methods is very low when compared with solid media.

Different culture systems utilising *in vitro* selection, have been successfully used by many workers. Some of these systems are: selection from callus culture (Chandler, *et al.*, 1986; He and Cramer, 1993); selection from suspension culture (Dix and Street, 1975; Merrick and Collin, 1981; Jain *et al.*, 1991); selection from plated cells (Meredith, 1978; Wong and Sussex, 1980a; Everret, 1981) and selection from protoplast cultures (Swanson *et al.*, 1988). These systems differ mainly in the way in which they are maintained. Each system has its own merits or otherwise, on the efficiency of selection.

Information on the cultural conditions favouring high frequency of *in vitro* regeneration is a prerequisite for the utilisation of these novel *in vitro* techniques (Jain *et al.*, 1988). A major limitation to the widespread application of tissue culture techniques to crop improvement is the lack of efficient regeneration protocols suited to plants of agricultural value. It is evident from many reports that, as with many other crop plants, regeneration is also possible in some species of *Brassica* through *in vitro* culture (Dunwell, 1981; Gupta *et al.*, 1990; Yang *et al.*, 1991; Hansen and Earle, 1994). Studies with several species of *Brassica* suggest an effect of genotype, choice of explant and composition of nutrient media on *in vitro* culture and regeneration (Dietert *et al.*, 1982; Jain *et al.*, 1988; Narasimhulu and Chopra, 1988; Yang *et al.*, 1991). Furthermore it has also been reported that each species required a particular hormone concentration for optimum growth and differentiation (Dunwell, 1981). A study of shoot regeneration from cotyledon explants of some diploid species of *Brassica* showed species-specific responses for *in vitro* shoot regeneration (Narasimhulu and Chopra, 1987). Not only the type of explant, the age, especially in case of hypocotyls and cotyledons, has also a great effect on the frequency of shoot regeneration (Yang *et al.*, 1991; Palmar, 1992). Taking in consideration these prerequisites, the initial part of this project studied the performance of different *Brassica* species in tissue culture, especially for regeneration. Investigations included an examination of explant source and media requirement in terms of hormone levels for callus initiation subsequent callus culture and regeneration.

### 1.3. Somaclonal variation

Breeding progress depends on selection within a variable population; selection may become ineffective when genetic variability is exhausted or falls below the level at which it can be detected (Gunn and Day, 1986; Bajaj, 1990; Grunewaldt and Dunemann, 1991). It has been known that plant tissue cultures undergo genetic erosion and show various changes, especially in chromosome number and ploidy level (Partenen, 1963; D'Amato, 1965; Murashige and Nakano, 1966; Karp and Bright, 1985; Karp, 1991). The term somaclonal variation, as defined by Larkin and Scowcroft (1981), is "variation displayed amongst plants regenerated from tissue culture". According to this definition somaclonal variation may be genetic as well as non-genetic. But some workers operate a restricted definition and consider only genetic variation as somaclonal variation (Amberger *et al.*, 1992; Bajaj, 1990). Somaclonal variation has been reported to occur for a range of agronomic traits such as yield, salt tolerance herbicide and disease resistance. (Daub, 1986; Larkin, 1987).

#### 1.3.1. Nature of variation

The phenotypic variation recovered in regenerated plants probably reflects both pre-existing cellular genetic differences and tissue culture induced changes (Skirvin and Janick, 1976; Orton, 1984; Evans, 1989). Pre-existing variation shown in tissue culture may be the result of mutation in somatic cells of the explant source. Such mutations do not express themselves in the whole plant and are not of use in traditional breeding. In tissue cultures, this variation may be exploited and multiplied and the plants regenerated from these mutated cells will show variation. In this way although tissue culture is not the source of inducing variation, ultimately it is the source of expressing and multiplying the hidden variation in the original cell population of the explant source.

The other type of variation i.e. variation induced by tissue culture can be further divided into three groups: (1) genetic (2) developmental (epigenetic) and (3) physiological (Challef, 1981; 1983; Berlyn *et al.*, 1990). Berlyn *et al.* (1990) suggested that the variation induced by the tissue culture may be a consequence of development, dedifferentiation, rejuvenation or redifferentiation, may be transient or may be persistent. The former two types of variations are called heritable cellular variations (which are transmitted from one cell generation to the other) while the physiological variations are non-heritable. The heritable cellular variations, generated by tissue culture cycles, could result from mutation, epigenetic changes or a

combination of both mechanisms (Meins, 1986). Genetic mutations result in genetic changes, such as those effecting nucleotide sequence of DNA and chromosome structure and number (Tal, 1990), which are sexually transmitted to the progeny of plants regenerated from cultured cells (Meins, 1983; Tal, 1990). Genetic variation is generally considered to be not readily reversible, stable through meiosis, and stochastic. One of the well documented examples of genetic variation is of a dominant, single-gene mutation (Meins, 1986). Some of other variation probably reflects changes in chromosomal constitution such as polyploidy, aneuploidy, and rearrangements (Sunderland, 1977; D'Amato, 1977; Bayliss, 1980).

The term epigenetic change denotes cellular heritable alterations, which are considered as a change in gene expression (Tal, 1990). They do not result from permanent changes in the cell genome (Harris, 1964). These changes differ from classical genetic mutation in several important ways. Epigenetic changes are 'transient' and potentially reversible. Epigenetic changes are often direct, i.e. they occur regularly in response to a specific inducer, and can be environmentally dependent. Epigenetic changes are not sexually transmitted. They are relatively stable throughout mitotic divisions and may, therefore, persist after the removal of the inducing conditions (Tal, 1990). In this way they are not of much importance for sexually propagated crops. On the other hand in case of vegetatively propagated crops epigenetic changes may be as useful as genetic change, if transmitted mitotically in a stable fashion. Physiological adaptation may also result from a change in gene expression due to some induced conditions and disappear with the cessation of the inducing conditions. This type of variation is called non-heritable variation and are not useful for crop improvement because they are not heritable from one generation to other.

### 1.3.2. Frequency of occurrence

From the work reviewed on somaclonal variation, it is clear that in general, the frequency of somaclonal variation is much higher than natural mutation (Meins, 1983). Scowcroft *et al.* (1985) reviewed a range of mutation frequency in somaclones, from less than 1% to 75% in different crops and different traits. Many other workers reported frequencies of 1-2% variation for any one character. From the studies on rice, Oono (1981) reported that the progeny of about 70% of somaclones differed from the parent for at least one heritable character. The possible reason for these wide range of differences in the frequencies of somaclonal variation, might be the effect of external factor(s) on somaclonal variation. The frequency of



somaclonal variation in the absence of known mutagenic agents is greatly influenced by factors such as genotype of plant, explant source, culture period and condition, type and level of growth regulators and cultural environment (Evans, 1986; Bajaj, 1990). Sometimes the recovery of cell culture mutants has been achieved at frequencies so high that known mutagenic treatments have often failed noticeably to enhance the frequency further (Larkin and Scowcroft, 1981). This is not so for all species, and indeed some species show very low levels of somaclonal variation and require the application of mutagenic agents to induce variability.

### 1.3.3. Genetic basis of somaclonal variation

The genetic basis of somaclonal variation observed in regenerated plants by many workers are: single gene nuclear mutations (Evans and Sharp, 1983; Edallo *et al.*, 1981; Fakuai, 1983), cytoplasmic genetic changes (Gengenback *et al.*, 1977, Kemble and Shepard, 1984), chromosomal aberration i.e. change in chromosome number (D'Amato, 1977; Orton, 1983), chromosome rearrangements, translocations (Ahloowalia, 1976; Shepard, 1982). Published work also suggests that chromosome deletions, additions, inversion, and crossing-overs also occur in regenerated plants (Meins, 1983; Evans, 1986; Scowcroft and Ryan, 1986; Grunewaldt and Dunemann, 1991). DNA methylation is also well documented as a source of genetic variation within somaclones (Brown, 1989; Muller *et al.*, 1990).

### 1.3.4. Somaclonal variation in *Brassica* species.

Somaclonal variation may be a valuable tool for the genetic amelioration of genus *Brassica* as this group includes several economically important vegetable and oilseed crop species. From the documented work it is evident that as with many other crop species, tissue culture in *Brassica napus* can be used to generate genetic diversity that may have value for genetic studies and plant improvement (Newell *et al.*, 1984). As compared with the plant population grown from the seeds, a wide range of variation has been observed among the progeny of *in vitro* regenerated plants of genus *Brassica* (Larkin and Scowcroft, 1981; Larkin *et al.*, 1985; Evans and Sharp, 1986). In this genus, variation has been observed among plants propagated from adventitious meristems of roots in cauliflower (Grout and Crisp, 1980) and plants regenerated from mesophyll protoplasts of brussle sprouts (Yamashita *et al.*, 1991). George and Rao (1983) observed yellow seeded variants amongst plants regenerated from cotyledonary explants of *Brassica juncea*. George *et al.* (1987) also reported variation for most of the agronomic characters, oil content and fatty acid composition in the anther derived plants of *Brassica juncea*. Jain *et al.* (1989) observed that a number of regenerated plants of *Brassica juncea* were agronomically

superior, high in oil content or early flowering as compared to their control counterparts. Sacristan (1981) reported change in chromosome number of *Brassica napus* L. plants regenerated from long term callus culture. Results from many studies suggests that change in chromosome number predominate amongst regenerants in *Brassica* species. (Horak *et al.*, 1975; Keller and Armstrong, 1978; Dunwell, 1981).

### 1.3.5. Somaclonal variation for salt tolerance

Improvement in any plant population requires the presence of genetic variability for the trait under consideration. Stress tolerance is no exception to this prerequisite. A genetic approach to the salinity problem is not very old, but has generated considerable interest worldwide. Since the beginning of 1980's attempts have been made to supplement conventional breeding toward the production of salt-resistant plants (Epstein *et al.*, 1980; Norlyn, 1980; Ramage 1980;) with variability derived from tissue or cell culture (Rains, 1982; Stavarek and Rains, 1984). The role of cell and tissue culture techniques in the development of salt-tolerant line has been emphasised by many workers. For instance, Dix and Street (1975) selected cell lines of *Nicotiana sylvestris* and *Capsicum annuum* at a lethal concentration (1% and 2% respectively) of NaCl. Similarly Hasegawa *et al.* (1980) selected a cell line of *Nicotiana tabacum* that was tolerant to 1% NaCl. Cell lines of *Medicago sativa* (McCoy, 1987) and *Oryza sativa* (Li *et al.*, 1987) also have been selected through tissue culture, which showed tolerance to 1% and 2% NaCl respectively.

Some of the questions raised by plant breeders and tissue culturists with respect to salt tolerance include concern about: (1) the extent of genetic variability as regards salt tolerance/resistance; (2) the means to increase this variability; and (3) the nature of this variability (Tal, 1990). The production of salt-resistant somaclones which transmit this characteristic to progenies have been examined successfully by many research workers (Nabors *et al.*, 1980; McHughen and Swartz, 1984; Jain (S) *et al.*, 1991a). However many workers have found this resistance is genetically unstable and lost when the plants were regenerated or even when the cells were grown under non-saline condition (Hasegawa *et al.*, 1980). These contradictory results leave many questions. Is this variability genetically stable? Is it epigenetic in nature or just physiological adaptation? These question still need to be answered. This study will attempt to answer some of these questions.

The amount and nature of potential spontaneous genetic variability for salt resistance in tissue culture is still not known. Salt resistance is considered as a



complex character (Ramage, 1980) which is controlled by nuclear as well as organelle genes (Tal, 1990). There is the possibility that, as with other complex characters, salt resistance may mainly be controlled by a few major genes with a range of minor modified genes. On the other hand Binzel *et al.* (1987) suggested that the multiple phenotypic and metabolic changes which characterise plant response to salt stress can be induced by a simple primary response, the genetic basis of which might be rather simple. The existence of genes having a major effect on salt resistance has been suggested as found in a fern (Hickok and Warnerr, 1987) and in pepper (Tal, 1985). The possible involvement of genes with a major effect on salt resistance is also indicated by molecular investigation of the gene products (protein) expressed under condition of salt stress when the expression was found to be stable in the absence of NaCl (Bressan *et al.*, 1987). Thus, it would appear that sufficient variability for salt resistance can be created in crop plants through tissue culture. Although we are not sure about the genetic nature of this variation, nevertheless it is still available for exploitation.

#### **1.3.6. General uses**

Somaclonal variation is now a well recognised phenomenon of possible value in crop improvement (Evans and Sharp, 1986). This tool, although not fully characterised, appears to present a useful source of genetic variation that could be of value to plant breeders. However, it may be thought that the technique will be of most value when the objectives of the breeding programme are concerned with characters that are expressed at a cellular level. Thus providing it is possible to create a suitable selective environment, somaclonal variation could be exploited effectively. Indeed the approach may be used as an alternative to the traditional backcross breeding programme for single gene characters. This always pre-supposes that it is possible to create a suitable selective environment to allow expression of new and valuable characters. As single gene mutations and organelle gene mutations have been produced by somaclonal variation, one obvious strategy is to introduce the best-available varieties into cell culture to select for improvement of a specific character. Hence, somaclonal variation could be used to uncover new variants that retain all the favourable qualities of an existing variety while changing one trait, such as disease resistance or herbicide resistance. However it is unlikely that only single character changes would occur, and other detrimental and agronomically unfavourable changes may also be produced along with the required favourable variations. Therefore the material may have to undergo further breeding activity.

Cellular selection is conceivable for the recovery of variants tolerant to antimetabolites such as amino acids analogues, antibiotic drugs, pathotoxins, herbicides, salts and physiological stress (Maliga, 1978; Thomas *et al.*, 1979; Brettell and Ingram, 1979). Variants so produced may be used as donor material to develop new varieties or parent lines (Evans *et al.*, 1984; Evans, 1986).

### **1.3.7. Detection of somaclonal variation**

One of the difficulties associated with somaclonal variation, has been the need to develop methods whereby material can be easily and rapidly screened to reveal any genetic differences from non tissue culture-derived controls.

The easiest and most commonly used method of screening is morphological analysis of regenerants. The first disadvantage of this approach is that possession of a normal phenotype is no guarantee that more cryptic changes have not occurred. Secondly many of the changes may be recessive and consequently, when in heterozygous forms, do not appear until plants have been selfed and the progeny examined (Göbel *et al.*, 1985; Brown *et al.*, 1993). Additionally, morphological variation shown by the regenerants may be epigenetic or just physiological and the status can not be confirmed, until progenies have been examined. This is exacerbated for some species in term of the length of the life cycle.

The other methods used for the detection of somaclonal variation are: gene product analysis; cytological and karyotypic studies; DNA fingerprinting or DNA profiling. Procedures such as protein and isozyme analysis provide a relatively convenient method for examining biochemical changes. The problems associated with these methodologies are that they may be subjected to alterations by environment and physiological development, and secondly variation shown will not confirm either genetic or non-genetic changes.

The methods mentioned above are based on phenotypic characters, or expressed products of genes. These characters may not be expressed uniformly, as expression may be related to environmental or physiological factors. In particular, only a relatively small proportion of genes present in the genome are actually transcribed and translated into protein at any given time, and environmental conditions, physiological age and the overall state of the plants can affect the expression of such character. These limitations have to the investigation of other methods of analysis based on the genome itself.

Karyological analysis of plants can show significant chromosomal changes such as alterations in ploidy levels as well as structural rearrangements (Karp and Bright, 1985; Karp, 1991). However, chromosomal changes cannot reveal alterations in individual genes (Brown *et al.*, 1993). More recently, methods to analyse the genome at the DNA level have been developed. The most convincing advantage of this type of methodology is the DNA sequence is essentially the same in all cells in all tissue of plants, and normally it is not affected by the physiological or the environmental conditions, and extracted DNA or plant parts/tissues can be stored for a long period of time by freezing.

DNA fingerprinting/profiling techniques appear to offer a unique opportunity to compare and contrast the effects of different tissue culture regimes in terms of effects on the genome (Potter and Jones, 1991). A precise determination of changes in a particular gene sequence resulting from tissue culture can be obtained by Restriction Fragment Length Polymorphism (RFLP) analysis. This methodology can allow comparison of genetic variation between callus and regenerants as well as among different ages of callus and plants. However, the method is limited in two main ways. Firstly the length of time required (usually 5-6 days) and secondly, the results of such an analysis is limited only to the gene sequence used as a probe (Brown *et al.*, 1993). It is also labour-intensive and requires the use of hazardous radioactive chemicals.

Development of a new technique using Polymerase Chain Reaction (PCR) with single random oligonucleotides for detecting polymorphism at DNA level, could provide an alternative technique for detecting variation at the DNA level. This technique is known as Random Amplified Polymorphic DNA (RAPD; Williams *et al.*, 1990). It has been well proven that these techniques could be easily applied to the determination of level of variation at all culture and growth stages (Brown *et al.*, 1993) and it circumvents many of the problems associated with RFLP.

#### 1.4. *In vitro* selection

Generally any progress, natural or artificial, which permits an increase in the proportion of certain genotypes in succeeding generations is termed as selection, but improving the genetic pattern of plants in relation to their economic use in plant breeding programs emphasises artificial selection (Frey, 1983).

*In vitro* selection must inevitably lead to the selection of cultured material which is more tolerant of *in vitro* conditions. However most workers usually use the term in relation to an additional stress imposed during culture. In practice both forms of selection must be occurring simultaneously (Grunewaldt and Dunemann, 1991). Progress, evaluation and selection normally begins as soon as the breeder has created a genetically variable population and continues until all important characters have been screened and if necessary fixed (Gunn and Day, 1986). Almost in all the studies using *in vitro* selection, the approach adopted is based on the assumption that plant cell cultures consists of a population of cells showing a range of genotypes (Collin and Dix, 1990). Only a few workers did not find sufficient variability for selection, and used artificial mutagenesis before selection (Cséplö *et al.*, 1985). *In vitro* methods for selection offer several important potential advantages over their *in vivo* equivalents. Each cell may be a potential plant and a large number of cultivated cells can be screened to select cells with the desired character. According to Grunewaldt and Dunemann (1991) selection *in vitro* covers two main advantages as compared with selection *in vivo*.

- The number of individuals simultaneously put under selection is high and nearly unlimited if single cell units can be used. Thus genotypes with a very low gene frequency in a population can be selected.
- The efficiency of *in vivo* selection methods is often too laborious and time consuming, or even non-existing, to select specific genotypes.

Other advantages of *in vitro* selection are:

- they can usually be applied at any time of the year;
- they often make it possible to maintain closer control of test condition;
- they avoid many hazards that are common in fields or glasshouse tests; and
- they may reduce the amount of space occupied by each test sample.

*In vitro* selection methods are a logical extension of research on the regeneration of plants from non-meristematic tissue culture where a selective agent is applied directly to material from which selected plants will be propagated.

Application of the selective agent may take place before, during or after plant regeneration (Gunn and Day, 1986). *In vitro* selection can be efficient and cost effective provided that regeneration of whole plant from selected cell(s) is possible and a possible correlation exists between the cellular and whole plant response to a specific selective agent. Genetic improvement for a character which can be identified at the cellular level such as enhanced resistance to an adverse factor in the cells micro environment might best be tackled by mutation and direct selection (Dix, 1986). But if sufficient variability is present, among the cell population grown in tissue, artificial mutation can be eliminated from the procedure.

The potential for the selection of traits of agronomic value using plant tissue culture has been well documented (Challef, 1983; Meredith, 1983). Cultured plant cells have been used for isolation of mutants *in vitro* with subsequent plant regeneration (Flick, 1983). The actual contribution that tissue culture has made to different fields of endeavour is variable but the ability to distinguish specific phenotypes at the cell level is a recurrent feature of the diverse fundamental and applied investigation in this area. For example, *in vitro* selection may lead to the production of plants showing resistance to specific environmental stresses, such as low temperature, salinity, drought, herbicides, toxic metals, and pathogens, and the selection of cell lines for analysis of the resistant mechanism at the cellular level (Collin and Dix, 1990). Promising results have already been obtained by selecting cultures for resistance to a host-specific pathotoxins (Gengenback *et al.*, 1977, Fadel and Wenzel, 1993), herbicide resistance (Challef and Parson, 1978a, Grant and McDougall, 1995) and salt tolerance (Jain *et al.*, 1991). Crisp and Gray (1979) described a successful application of *in vitro* selection system to produce potential new cultivars for curd quality in cauliflower.

#### **1.4.1. Selection strategies.**

Various approaches can be developed to affect selection of particular kind of variant. The strategy used for selection depends on the nature of the desired characters. Although only resistance selection has been adopted throughout this research programme, the principles and a brief introduction of other selection strategies is presented for information and comparison.

##### **1.4.1.1. Resistance selection.**

This is certainly the most widely practised approach to selection *in vitro* (Dix, 1986; Collin and Dix, 1990). It is applied whenever conditions can be defined under

which the required phenotype has a selective advantage over wild type cells. The mass culture is simply incubated under the selective conditions (e.g. toxic level of herbicides, salt.). In principle only resistant cells should grow, and hence the resistant lines can be recovered. This approach is applicable not only to the selection of tolerant variants (Maliga, 1978), but also to the selection of other classes, such as lines capable of growing without added growth hormones (Meins and Binns, 1977; Everret, 1981) or with alternative carbon source (Challef and Parson, 1978b).

#### 1.4.1.2 Visual selection.

This is applied when the required phenotype has no selective advantage over wild type cells (Collin and Dix, 1990). Most commonly it applies to accumulation of a pigment, such as anthocynin (Dougall *et al.*, 1980) or loss of a pigment, such as chlorophyll (Svab and Maliga, 1986).

#### 1.4.1.3. Counterselection.

For many conditional lethal mutants, such as auxotrophs, there is no straightforward approach to selection (Collin and Dix, 1990). Counter selection is an approach which has been developed to circumvent this problem. It is based on the fact that certain drugs will kill only dividing cells, so that others, division of which is inhibited, will survive. In this type of selection sometimes enrichment techniques are integrated. Specific chemicals are then added to the culture medium, which kill only the dividing cells. This method is also called negative selection (Grunewaldt and Dunemann, 1991).

#### 1.4.1.4. Total selection.

Total selection is referred to the systematic screening of large number of cell colonies for non-selectable characters (Collin and Dix, 1990). For cells, phenotypes not readily amenable to selection procedures total selection can applied. This involves screening of a large number of individual colonies, applying appropriate test to establish phenotypes. Due to the tedious nature of this selection strategy, it was not employed to obtain higher plants auxotrophs until 1979 when Savage *et al.*, recovered a pantothenate-requiring line of *Datura innoxia*. Similar methods have been used to obtain adenine-requiring lines of *Datura innoxia* (King *et al.*, 1980), and other conditional lethal mutants in *Hyoscyamus muticus* (Strauss *et al.*, 1981; Gebhardt *et al.*, 1981) and *Nicotiana plumbaginifolia* (Sidorov *et al.*, 1981). For the isolation of auxotrophs total selection is the alternative strategy to counterselection and may lose favour if highly effective counterselection procedures are developed. This method is also useful for the screening of cell lines producing secondary metabolities, which can



not be visually distinguished. But in this case selection is based on chemical analysis rather than growth tests.

#### 1.4.2. Choice of material

The selection of plant cell material from which to isolate mutants may determine the success or failure of *in vitro* selection. It may also determine the stability of the character and regenerative capacity of the culture. The material available for selection may vary from single cells (or even protoplasts) to complex differentiated tissue. The choice of species and explant source material is often determined by availability of the material, interest of the worker and response to *in vitro* culture. The limitations on the choice of starting material depend on the character, the nature of the cell line being selected and the selection strategy adopted (Dix, 1986). In evaluating the use of different types of tissue culture for selection, their use with reference to the recent literature could be illustrated, in keeping with the continuing development of this area.

##### 1.4.2.1. Callus cultures

Callus cultures are probably the most frequently used tissue culture system for direct selection of mutants because callus is the easiest undifferentiated material to which a selection pressure can be applied. Callus consists of a mass of tissue with no or a low level of organisation. It is obtained by transferring a suitable piece of plant tissue to a suitable nutrient medium and maintained indefinitely by regular transfer to fresh culture medium. In such a selection procedure, callus aggregates are placed on media containing the selective agent. Callus pieces which show further cell division are isolated. These growing segments are bulked-up and re-tested on the selective medium (Dix, 1986). The criticisms of the use of callus in a selection procedure are, the potential risk of producing chimeras in both callus and derived plants (Collin and Dix, 1990; Grunewaldt and Dunemann, 1991); all the cells in a callus piece are not uniformly exposed to selective agent (Flick, 1983) and susceptible cells may survive due to cross-feeding. Alternatively, resistant cells may not be able to grow if surrounded by large number of sensitive cells which are not growing (Flick, 1983). One way of reducing these risks is to use very small pieces of callus. For example, Wakasa and Widholm (1987) used 10-20 mg callus piece in selecting for 5-methyltryptophan resistant rice lines. In screening for salt tolerance in *Brassica napus* callus pieces of 20 mg fresh weight were transferred to selective medium (Chandler and Thrope, 1987).

The selection procedure has been based on either a direct or a stepwise approach. Most of the selections involved the direct approach, where the callus was exposed to one concentration of selective agent, then the surviving cells isolated and bulked. This procedure was adopted for the selection of resistance of salt (McHuguen and Swartz, 1984; McHuguen, 1987; Chandler and Thrope, 1987), amino acid analogues (Wakasa and Widholm, 1987; Schaeffer and Sharpe, 1987), herbicides (Jordan and McHuguen, 1987), oxalic acid (Chunren and Houli, 1991), and heavy metals (Khadeeva *et al.*, 1985).

The alternative approach is to use the less favoured but equally effective stepwise approach (Collin and Dix, 1990) where the concentration of the selective agent is gradually increased and the surviving callus is sequentially transferred to increasing level of selective agent. Problems associated with this approach may be that there are more chances of physiological adaptation. Pua and Thrope (1986) transferred callus of *Beta vulgaris* from 30 mM Na<sub>2</sub> SO<sub>4</sub> with a stepwise increase of 15 mM at each 3 week culture period to 210 mM Na<sub>2</sub> SO<sub>4</sub>, and tobacco callus resistant to 2,4-dichlorophenoxy acetic acid was obtained in the similar manner (Nakamura *et al.*, 1985).

#### 1.4.2.2. Cell suspension cultures

Cell suspension cultures offer improved possibilities to regenerate homogeneous plants from small cell aggregates or even single cells treated with a selective agent (Grunewaldt and Dunemann, 1991). A cell suspension which is well dispersed provides a suitable culture for selection since the single cells and small cell aggregates, are uniformly exposed to the selective agent. Cell suspension cultures are most commonly obtained by dispersing friable callus in a liquid medium, and agitating on a rotary shaker, although they can also be initiated directly from explants by treating with a suitable enzyme. The degree of dispersion, and growth rate of suspension cultures, are dependent on the species, culture conditions, type and concentrations of growth regulators, explant source material and the friability of callus from which the suspension is initiated. One problem associated with cell suspension cultures is the size of cell aggregates. Large cell clumps may present the same problems as are encountered with callus selection and such cultures would require periodic removal of such aggregates by filtration (Flick, 1983). Single cells and small cell aggregates are then exposed to the selective agent to select the resistant cell(s). A further problem in suspension cultures is regeneration of plants. As cells in fine suspension cultures have no organisation, redifferentiation and regeneration is more difficult and regenerative



ability is less as compared with callus cultures. Finally cells exposed to suspension culture for long periods lose their ability to undergo redifferentiation.

As the initiation and establishment of cell suspension cultures are critical steps with respects to the inoculum density and cell multiplication, mostly a stepwise increase of the selective agent concentration has been chosen (Grunewaldt and Dunemann, 1991; Collin and Dix, 1990). Since the increases in the selective agent are relatively small, in the initial stages there are always a number of cells in the inoculum which can tolerate the new concentration. But in this way it takes more time as compared with that one step selection, in which cells are directly exposed to the selective concentrations.

#### 1.4.2.3. Plated cells

Because of the low regenerative potential of cell lines produced in long term liquid cultures, methods combining liquid and solid culture have been thought to provide a more promising approach (Grunewaldt and Dunemann, 1991). In this method a fine suspension culture is established initially and then the cells are transferred to a petri dish on to or in a solid medium containing the selective agent. The purpose of the plating step is to expose the single cells, and small cell aggregates from the suspension, to the selective agent, and then to be able to identify and remove individual resistant colonies. One practical advantage of the plating technique is that the screening can be carried out on a large scale with minimum space requirements. The products can also be very easily visualised (Collin and Dix, 1990).

The most widely used methods is to mix a portion of the cell suspension with molten agar medium which is then poured into petri dishes (Dix, 1986). Alternatively, all suspensions may be spread or sprayed on to the agar surface or incorporated into other matrices. For example Corner and Meredith (1985a), while selecting for aluminium resistance in *Nicotiana plumbaginifolia*, dispersed a small volume of cell suspension over filter paper supported by polyurethane foam and saturated with medium containing aluminium salts. In a similar approach when selecting for aluminium tolerance in potato cell cultures, Wersuhn *et al.* (1986) transferred cell suspension to petri dishes containing liquid medium with filter paper to support the cells. The liquid medium containing aluminium was replaced every seven days.

One of the drawbacks of plated cells is the prolonged growing period, other than that it does not seem to have particular problems. Therefore this technique has been used to produce a large number of very diverse phenotypes including antibiotics (Sung, 1976), nucleotide base analogue (Ohyama, 1976; Sung, 1976), abscisic acid (Wong and Sussex, 1980b), mercury (Collin *et al.*, 1979), and aluminium (Meredith, 1978) resistance, 2,4-D independence (Everret, 1981) and auxotrophy (Savage *et al.*, 1979).

#### 1.4.2.4. Protoplast cultures

Ideally an *in vitro* selection programme should be based on single cells since then each cell is exposed uniformly to the medium and could give rise to a discreet colony, thus avoiding the cross feeding of the cells and the production of chimeras. Protoplast culture may lead to a reduced level of chimeral mutants (Collin and Dix, 1990; Grunewaldt and Dunemann, 1991). Although protoplasts can now be isolated from many species, such cells are not used routinely in cell selections. The reasons for this limited attention for many species are that the yields of protoplasts are often too low, the viability of the isolated protoplasts may be variable and the procedures for initiating wall formation and division are often complex and not always successful. Direct selection at this stage adds yet another level of manipulation to an already complex procedure (Collin and Dix, 1990). These problems have not discouraged some investigators. Usually protoplast-derived colonies are particularly attractive for total selection or the selection is delayed until cell division has commenced. Muller *et al.* (1985) isolated protoplasts from tobacco mesophyll and exposed then to a selective agent five days after isolation. Cséplö *et al.*, (1985) exposed mesophyll protoplasts from *Nicotiana plumbaginifolia* to a mutagenic agent and allowed cells to grow up to the colony stage. These were then plated in medium containing the triazine herbicide and tolerant colonies were selected.

#### 1.4.3. *In vitro* selection for salt tolerance

Salinity is a significant limiting factor to agriculture productivity. Epstein (1980) estimated approximately  $9 \times 10^8$  hectares of the land surface on the earth, an area about 3 times greater than all of the land that is presently irrigated, affected by salinity. The FAO (1990) reported that about 20 to 30 million hectares of irrigated land world wide, are severely affected by salinity and an additional 60 to 80 million hectares are affected to some extent. Estimates of the area affected by salinity have ranged from 10-48% of total irrigated land area world wide (Umali, 1993). According to an other estimate the rate of loss of agriculture land, world wide, is 5-7

million hectare per year and overall salinisation is the second major cause of such losses (Umali, 1993). Similarly in Pakistan, soil salinity is one of the most common stress problems, in crop production (Ashraf, 1994). It has been estimated that 21% of total irrigated land is affected by salinity in Pakistan (Umali, 1993). According to another estimate, in Pakistan, out of 14.5 million hectares, approximately 6 million hectares of irrigated land is affected by salt (Aslam *et al.*, 1993; Qurashi *et al.*, 1993). It is evident that high salt concentrations in soils cause growth inhibition in most crop plants. Salinity retards almost all growth process of plants, that have been studied, including cell division, cell enlargement; protein and nucleic acid production (Maas and Nieman, 1978). Generally soil salinity is due to a mixture of different salts and ions present in excess which have specific effects on plants (Maas and Nieman, 1978). Excess of sodium chloride is the most wide spread chemical condition in saline soils inhibiting plant growth (Rains and Epstein, 1967).

There are two possible ways of utilising such unproductive land: (1) by reclaiming affected soils and (2) by producing resistant genotypes capable of cultivation on these soils. The cost of modifying the environment to suit the plant through, for example, irrigation, fertilisation, soil modification, provides a strong argument for the development of plants with increased tolerance to salt. Salt tolerance has been defined as “the ability of plants to grow satisfactorily on saline soils” (Ashraf, 1994), or “sustained growth of plants in an environment of NaCl or combination of mixed salts (Levitt, 1972)”. Development of a simple, rapid and reliable screening method is needed however, to provide breeders with a tool for selecting plants with improved response to salt stress. Conventional breeding schemes have met with limited success in improving the response of crops to saline stress (Epstein *et al.*, 1980). Application of plant tissue culture techniques has provided another method to develop salt-tolerant genotypes (Meredith, 1984; Stavarek and Rains, 1984; Dix, 1986; Jain *et al.*, 1986). Cell culture techniques, as supplementary procedures, may enhance the potential for achieving this goal. The environment and nutrient conditions can be controlled uniformly and precisely and a large number of cells can be screened rapidly in a small volume/area.

The potential use and problems associated with the application of cell culture methodology for the selection of stress-resistant cell lines and for studying the cellular response for stress resistance has already been discussed by many workers (Nabors, 1976; Croughan *et al.*, 1981 Stavarek and Rains, 1984; Chandler and Thrope, 1986). There are real prospects for the production of salt resistant cultivars of crop plants

through *in vitro* selection. Studies on NaCl-resistant and sensitive cell lines can help in the elucidation of the cellular components of salt stress injury and resistance (Dix *et al.*, 1986). Tissue culture techniques have been used to obtain salt-tolerant genotypes in several plant species including *Brassica* species (Jain, 1991). Jain *et al.*, (1986) screened NaCl resistant lines of *Brassica juncea* L. through *in vitro* selection. The techniques used, were based on isolating and multiplying saline-tolerant cells which might have arisen as a result of spontaneous mutations in the somatic tissues. Since all the sensitive cells died and only the resistant ones grew to form calli and subsequently whole plants, this selection offered a potential tool for screening somatic mutations tolerant to salinity. Further studies were carried out to check the stability of the selected traits (Jain *et al.*, 1991). Selected plants were multiplied and maintained on NaCl-free medium for a total period of three months before bringing back on to stress medium. All the selected salt-tolerant shoots grew on medium containing NaCl, whereas the control shoots as well as seedlings turned brown and died.

One of the limitations of the use of tissue culture to develop salt tolerance is that correlation of salinity tolerance of a plant with that of cultured cells occur only if tolerance of the plant is due predominantly to cellular based mechanisms rather anatomical structures or physiological organisation (Orton, 1980; Smith and McComb, 1981; Warren and Gould, 1982). Chandler *et al.* (1986) indicated, an at least partially-common basis to relative Na<sub>2</sub>SO<sub>4</sub> sensitivity *in vitro* and *in vivo*. On the basis of other examples where salt tolerance in tissue culture is correlated to salt tolerance at whole plant level (Orton, 1980; Smith and McComb, 1981; Warren and Gould, 1982), the common component is presumably metabolic and not morphological. If variability in such components is under genetic control then selection of salt tolerant cell lines may be a means of producing plants resistant/tolerant to salts (Chandler *et al.*, 1988b).

### 1.5. Aims and objectives

The overall aim of the study was to examine the feasibility of *in vitro* callus selection as an alternative to selection in conventional breeding programme. The intention is to use the system as model, not to produce *Brassica* lines resistant to stress condition(s).

By using *in vitro* callus cultures and selection the aims are to study the effect of genotype, explant sources and growth regulator concentrations on callus induction, subsequent callus cultures and plant regeneration. To study the effectiveness of *in vitro* callus selection for crop improvement with specific reference to salt tolerance. To study the stability of selected callus, to salt tolerance. To investigate and estimate general variation that occurs in somaclone. And finally to appraise this approach in plant breeding.

## 2. MATERIALS METHODS

This chapter will describe materials and methodology in general. Specific methodology relating to specific experiments will be described in the later results sections. Addresses of suppliers of important equipment, material, chemicals, reagents, and kits is presented in appendix 2.1.

### 2.1. General conditions

The research work was conducted in the plant growth unit and laboratories of the department of Crop Science and Technology, Scottish Agricultural College, Edinburgh. Plants were grown in glass houses at  $22\pm 2^{\circ}\text{C}$  under 14/10 hours light/dark cycle. The light source used were 400 watt, high pressure, sodium lamps ( $\approx 10000$  lux). All *in vitro* cultures were incubated in the culture room at about  $25^{\circ}\text{C}$  under 16/8 hour light /dark cycle. In culture room the light was provided by 70 watt, cool, white fluorescent light ( $\approx 4500$  lux).

All *in vitro* cultures and preparation were done inside a laminar flow cabinet (Bassair). All the equipment used was sterile and every effort was made to prevent contamination of cultures. Petri dishes were sealed with double layer of parafilmM. Experimental design used in most of the experiments, was Completely Randomised Design (CRD). Statistical analyses were carried out using Minitab Statistical Software, standard version, release 9.1 and Genstat 5, release 2.2.

### 2.2. Plant material

Three species of genus *Brassica* were chosen to study in this project. In selecting these species two points were taken into consideration. (1) The species should reflect the genome diversity and ploidy level of the *Brassica* group. (2) Given the aim to effect to selection in cell lines, the material should reflect a range of genetic constitutions, homozygosity to heterozygosity. Therefore a single type of genetic composition was avoided and the species selected were from three different types of genotypes i.e. F1 hybrid, inbred line or pure genetic material/breeder's material and field cultivated variety. The species studied in this project are as below.

***Brassica oleracea* L. F1 generation of curly kale (Bronick F1).** Supplied by Nickerson Seeds . Genome cc, n=9.

***Brassica napus* L.**     **Winter oilseed rape var. Apache.** Provided by Mr. Robert Redpath. Already present in the department, as a sample from Official Seed Testing Station, Cambridge. Genome aacc, n=19.

***Brassica rapa* L.**     **Rapid cycling species, Genome aa.** Supplied by Crucifer Genetics Co-operative, USA. n=10.

### 2.3. Preparation of culture media

Culture media used in this project consisted of Murashige and Skoog (1962) macro and micro nutrients (Table 2.1), supplied by Flow Laboratories. Culture media was supplemented with varying concentrations of growth regulators specific to requirements, sucrose as carbon source and Phytogel™ (Sigma®) to solidify the medium. The concentrations of sucrose and phytogel used were 3% and 0.2% respectively, unless otherwise stated.

Recommended (or proposed) amount of media and sucrose was mixed in ¾th of total quantity of distilled water and agitated on a gyratory shaker to dissolve the material completely. Growth regulators were added in the solution and made up to the required volume by adding distilled water. The pH of the media was adjusted by 0.1 M NaOH and 0.1 M HCl, to a range 5.6-5.8. The Phytogel™ was added into the media just before autoclaving. The media was sterilised by autoclaving (Drayton Castle model) at 1 kg cm<sup>-2</sup> pressure and 120°C for 20 minutes. The media was kept warm at 50°C until pouring in the culture pots. The media was dispensed into sterile pots under sterile conditions in a laminar flow cabinet and allowed to cool and set before culture.

### 2.4. Explant sources and their preparation

Four types of explants i.e. hypocotyls, petioles, leaf discs without main vein and leaf discs with vein, were used in different studies. Hypocotyls were taken from *in vitro* grown seedling while petioles and leaf discs were taken from the glasshouse grown plants. Seeds (for hypocotyl explants) were surface sterilised before sowing while petioles and leaf lamina were surface sterilised at the time of explant preparation.



#### **2.4.1. Surface sterilisation and sowing of seed**

Seeds were surface-sterilised by submerging in 70% ethanol for 2 minutes followed by rinsing with sterile distilled water. The seeds were then immersed in 4% sodium hypochlorite solution containing one drop of Tween-20 per 100 ml for 20 minutes. The seeds were rinsed thoroughly, 3 times, with sterile distilled water and allowed to dry inside the laminar flow cabinet. Surface-sterilised seeds were sown aseptically on  $\frac{1}{2}$  strength MS-medium supplemented with 1% sucrose and 0.2% Phytogel™.



Table 2.1. Composition of MS-medium used in tissue culture.

<b>Major Nutrients</b>	
$\text{NH}_4\text{NO}_3$	1650
$\text{KNO}_3$	1900
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370
$\text{KH}_2\text{PO}_4$	170
<b>Micro Nutrients</b>	
KI	0.83
$\text{H}_3\text{BO}_3$	6.2
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	22.3
$\text{Zn}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$	8.6
$\text{Na}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$	0.25
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025
$\text{Na}_2\text{EDTA}$	37.3
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.8
<b>Vitamins and Additives</b>	
Inositol	100
Nicotinic Acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Glycine	2.0

All figures in  $\text{mg l}^{-1}$

Media were dispensed in 60 ml specimen containers (Sterlin™). Three seeds were sown in each container. Germination was carried out in the culture room.

#### 2.4.2. Sterilisation of leaves and petioles

To take leaves and petioles as explant source material, seeds were sown in Shamrock compost in plastic seeds trays. After 15 days healthy seedlings were transferred to pots containing the same compost. The plants were grown-on in the glasshouse. Expanded leaves and petioles, from about one month old plants, were removed. Leaves were cut into 3-4 pieces. Excised plant material was rinsed in 70% (v/v) ethanol with gently rubbing. After washing with sterile distilled water the plant material was submerged in 2% sodium hypochlorite and agitated on a gyratory shaker for 15 minutes. Finally the plant material was rinsed several times with sterile distilled water to remove the traces of sodium hypochlorite, and blotted on sterile tissue paper. The surface-sterilised leaves and petioles were used as explants for callus initiation.

#### 2.4.3. Preparation of explants

The age of the hypocotyl is very critical when used as explant source, for *in vitro* cultures and particularly for regeneration. Yang *et al.* (1991) studied *in vitro* regeneration frequencies from hypocotyl explants of *Brassica campestris* and reported that high frequency shoot regeneration occurred only from explants originating from 6-7 day old, but not younger or older seedlings. Lentini *et al.* (1986) observed maximum callus induction from 3-6 d old hypocotyls. About the same aged hypocotyls have been used successfully by other researchers as well, such as Murata and Orton (1987) (7 day); Msikita and Skirvin (1989) (7 day); Gupta *et al.* (1990) (5-6 day). From these reports it would appear that it is better to use hypocotyl of about 6 day old and this became standard practice in this project.

The size of the hypocotyl segment, used as explant, is also important to successful callus initiation and regeneration in cultures. Several reports are available describing the hypocotyl segments size used as explants, including Murata and Orton (1987) (10 mm); Yang *et al.* (1991) (6-7 mm); Bajaj and Nietsch (1975) (10 mm); Msikita and Skirvin (1989) (10 mm). On the basis of these reports it was decided that for this project hypocotyls would be cut in about 10 mm segments to prepare explants.

Hypocotyls were collected from 6 day old *in vitro* grown seedlings. Roots and cotyledons were removed with the help of a fine, sterile scalpel. Axillary bud-free

hypocotyls were placed into a sterile, plastic petri dish of 90 mm diameter, and cut transversally into approximately 10 mm segments.

Surface-sterilised leaves were used to punch out the leaf discs for culture. Small pieces of the sterile leaves were placed into 90 mm diameter, sterile petri dishes. Discs were punched with the help of sterile, metal cork borer with internal diameter of 6 mm. Different types of the leaf discs were cut separately.

Sterile petioles of about equal thickness were placed into 90 mm diameter petri dishes. The cut surface of the petioles, which were directly exposed to the sodium hypochlorite during sterilisation process, were removed with the help of a fine scalpel. The rest of the petioles were cut into approximately 10 mm long segments and used as explants for callus initiation.

## **2.5. Callus initiation**

All type of explants were cultured on MS-medium for callus initiation. Hypocotyl and petiole segments were placed horizontally on the surface of the culture medium and pressed slightly to make contact with the medium. Three pieces of explants were placed in each petri dish of 50 mm diameter.

Leaf discs with and without main vein, were carefully placed on surface of the culture medium keeping adaxial surface up. The discs were pressed slightly to make contact between the medium and abaxial surface of the leaf discs. Three leaf discs of the same type were placed in each petri dish of 50 mm diameter.

The petri dishes were sealed with double layer of parafilm. all the petri dishes were placed randomly in the culture room and incubated for one month.

## **2.6. Sub-culture and maintenance**

Sub-culturing becomes imperative to maintain the culture or to increase volume of callus, because the growth of cells in a limited environment leads eventually the accumulation of toxic metabolites and exhaustion or drying-out of the medium. Therefore, callus cultures require to be transferred periodically to a fresh nutrient medium for continuity of growth, the length of which depends on the rate of cell growth or genotype in use. Once well established, most callus cultures lines will

require regular sub-culture at approximately 4-week intervals (Dixon, 1985). In case of *in vitro* culture of *Brassica* species different workers sub-cultured callus with different intervals ranging from 3-6 week intervals e.g. Gupta *et al.* (1990) (3-4 weeks); Hegazi and Matsubara (1991) (Monthly); Chandler *et al.*, (1986) (4-6 weeks and 3 weeks). For this project it was decided that callus sub-cultures would be made with 4-weekly intervals.

All the cultures were removed from the petri dishes and only healthy-looking callus pieces were sub-cultured on the new media. Callus pieces were divided into small pieces according to the requirement and transferred to the surface of fresh media in 50 mm diameter, single vent, deep (Sterlin™) or 90 mm diameter (Media™) petri dishes. All the cultures were incubated in the culture room with the conditions given above. Each culture was named with letter "C" followed by a number showing the number of cultures e.g. "C3" means the third culture cycle of one month each.

## 2.7. Assessment of callus growth

Growth of callus cultures has been measured by many ways, such as callus fresh weight (Turner and Dickinson, 1993); percent-increase in callus fresh weight (Chandler *et al.*, 1986; Jain *et al.*, 1990); relative increase in callus fresh weight (Chunren and Houli, 1991; He and Cramer, 1993); callus dry weight (Bressan *et al.*, 1985); automated image analysis (Nyange, 1994) and callus score based on area covered (Dietert *et al.*, 1982; Ogihara and Tsunewaki, 1979). While selecting appropriate methods emphasis was given to non-destructive and reliable techniques. Therefore generally callus fresh weight was selected to measure the growth. Callus growth from hypocotyl explants was measured as final fresh weight of callus produced and callus yield from sub-cultures was measured as relative increase in callus fresh weight. The reason for using the relative increase in callus fresh weight in case of callus culture was that, without weighing, it was impossible to maintain equal initial callus weight in all petri dishes. Variations in the inoculum weight, in different petri dishes, may effect the callus growth. Therefore to minimise the effect of the variation in the initial callus weight, on callus growth, initial fresh weight was also taken into account and the growth was measured as relative increase in callus fresh weight.

To calculate the callus fresh weight (Wf), petri dishes were weighed along with callus pieces (W1), just before harvesting of the callus. The callus pieces were

removed carefully from the petri dishes, leaving all the culture medium inside and the petri dishes were weighed again (W2). The callus fresh weight, in each petri dish, was calculated (W1-W2). Direct weighing of the callus was avoided due to the risk of contamination because the same callus was used for sub-cultures and further studies. To calculate the initial callus fresh weight (Wi), petri dishes were weighed before (W1) and after (W2) the inoculation with the callus piece, and W2-W1 gave initial fresh weight.

To calculate the relative fresh weight gain, initial fresh weight of callus inoculated (Wi) and final fresh weight produced (Wf) were measured as mentioned above. The relative fresh weight gain was calculated as  $(W_f - W_i)/W_i$  (He and Cramer, 1993).

## 2.8. *In vitro* selection, maintenance and stability tests

Normally selection of the callus for NaCl resistance was started at the time of fourth sub-culture. This was chosen to allow the callus proper time for the multiplication of material in sufficient quantity and to give the callus some time for the accumulation of somaclonal variation. Callus cultures maintained by continuous subculturing, were cut into small pieces ( $\approx 60$  mg). Five pieces of callus were picked randomly and cultured in each petri dish, on selective media for selection and on non-selective media for control.

After one month of incubation on selective medium callus pieces or parts showing growth and looking healthy, were separated and again cultured on selective medium to reduce the effect of cross feeding on resistance. After one month cultures were again observed and the whole callus pieces or parts still growing, were selected and sub-cultured to normal culture media for maintenance. Selected callus were maintained on normal culture medium by monthly sub-culturing until required for further studies.

Selected callus showing significantly more NaCl tolerance as compared with the un-selected callus, even after culturing on salt free medium, was considered as stable for NaCl tolerance. To assess stability, the selected and maintained callus pieces were transferred to selective media and growth was checked after one month of incubation. In all the cases growth was measured as relative fresh weight gain (2.7). A tolerance index was calculated as described by Turner and Dickinson, (1993).

$$TI\% = \frac{Gs}{Gc} \times 100$$

Where *TI* stand for tolerance index, *Gs* is for growth on selective medium and *Gc* for growth of the same callus, on control (non-selective) medium.

Each selection and maintenance cycle was abbreviated with the letter S and M respectively, with an appropriate number. The number before the abbreviation indicates the numbers of culture cycles completed in the particular phase and the number after the abbreviation indicates the current number of cycles in the particular phase. For example “C7 2S M3” mean, culture is in seventh cycle, has been selected for two cycles and is in 3rd cycle of maintenance after selection.

## 2.9. *In vitro* regeneration

To regenerate plants from callus pieces, the callus pieces were transferred to a regeneration media in 60 ml specimen containers and closed with plastic screw caps. Regenerated multiple shoots were separated and transferred again on the fresh regeneration media in reusable glass jars or disposable 150 ml specimen containers. Shoots were allowed to grow in the same culture conditions. Regenerated shoots of suitable length and shape were transferred to the rooting media in the glass jars. Shoots were allowed to incubate in the culture room until the sufficient roots were developed.

## 2.10. Hardening and transfer of plants

Epicuticular wax, that helps to control transpiration, is lacking or present in reduced quantity in the plantlets regenerated *in vitro*. Consequently, the regenerated plantlets frequently show symptoms of severe water stress when transferred to an open bench in a glasshouse (Grout and Aston, 1977). Control of the humidity around the plantlets following its transfer from the water-saturated atmosphere of the culture tubes, was critical for survival, and special treatments were needed to acclimatise the regenerated plants to the new environment. The treatments given to the regenerated plants to help in acclimatisation in the changed environment, included covering with plastic covers (propagators) for 1-2 weeks to provide high humidity environment around the plantlets.

Regenerated shoots with sufficient roots were removed from the jars. Roots were washed thoroughly in running tap water to washout all the media. Roots were handled very gently. Regenerated plants with washed roots were transferred to compost in disposable plastic pots. Pots were placed in plastic trays and flooded with water. Plants were covered with propagator for hardening and the trays were placed in the glasshouse. Plants were kept in hardening condition for 15 days. Then the covered propagators were removed and the plants were allowed to grow in normal glass house conditions. When the plants were acclimatised with new culture conditions and big enough, they were transferred to 23 cm plastic pots. Plants were watered regularly.

## **2.11. Bio-molecular analysis**

For bio-molecular analysis, tissue samples and regenerated shoots were collected from different culture cycles and conditions. Collected samples were dipped into liquid nitrogen for few seconds, and stored in glass vials at  $-80^{\circ}\text{C}$  until they were needed for the analysis. Different samples were stored in different vials which were clearly marked, showing the culture cycles and type of the sample.

### **2.11.1. DNA isolation**

DNA from different samples was isolated using Puregene® DNA Isolation Kit (Gentra System Inc.). The kit was consisted of four solutions named as Cell Lysis Solution, Protein Precipitation Solution, RNase-A Solution and DNA Hydration Solution (Appendix 2.2). One gram of fresh or frozen plant material was ground into fine powder in liquid nitrogen, with the help of a sterile mortar and pestle. Powdered material was transferred into 50 ml sterile centrifuge tubes. Eighteen ml of Cell Lysis Solution was added into the tubes and homogenised using 30-50 strokes with a tube pestle. Samples were incubated in a water bath at  $65^{\circ}\text{C}$  for 60 minutes.

To remove the RNA from the lysate, tubes were cooled to room temperature and 90  $\mu\text{l}$  of RNase-A solution was added in the tubes. The tubes were inverted 25 times to mix the samples and incubated at  $37^{\circ}\text{C}$  for 15 minutes. The tubes were again cooled to room temperature and treated with 6 ml of Protein Precipitation Solution. Samples were vortexed vigorously at high speed for 20 seconds to mix the solution uniformly. Tubes were centrifuged at  $3000 \times g$  for 5 minutes. The precipitated protein formed a tight brownish green pellet at the base of the tubes. To precipitate the DNA only the supernatant was poured carefully in to a 50 ml centrifuged tube containing 18 ml 100% isopropanol. Tubes were inverted 50 times to mix the



solutions and centrifuged at  $3000 \times g$  for 5 minutes. At this stage the DNA made a visible pellet at the base of the tubes. Supernatant was poured off and the tubes were drained on clean absorbent paper.

To wash the DNA pellet, 18 ml of 70% ethanol was added and the tubes were inverted several times. Tubes were again centrifuged at  $3000 \times g$  for 5 minutes and the ethanol was poured off carefully. Tubes were drained by inverting on absorbent paper and DNA was allowed to air dry for 15 minutes. Subsequently the DNA was rehydrated by adding 1 ml of DNA Hydration Solution. Tubes were left over night on a shaker at room temperature or shaken in water bath at  $60^{\circ}\text{C}$  for one hour, depending on the time available. Rehydrated DNA was again centrifuged at  $3000 \times g$  for 5 minutes to remove particulate from the dissolved DNA. Supernatant was pipetted out into a 1.5 ml tube and stored at  $4^{\circ}\text{C}$ .

### 2.11.2. DNA visualisation

To confirm a successful extraction, isolated DNA was visualised with ethidium bromide after electrophoresis on a 1.4% agarose gel, by adopting the standard protocol of Sambrook *et al.* (1989). Gels were prepared in  $1 \times$  TBE (Tris-HCl, borate, EDTA)-buffer. The required quantity of agarose (Bio-Rad) was added to the TBE buffer (Supplied by Sigma in  $5 \times$  concentration) and microwaved to dissolve completely. Once cooled to about  $50^{\circ}\text{C}$  the solution was poured in to a gel tray containing a comb. The agarose gel was allowed to set and then the comb was removed. The gel was then submerged in running buffer (TBE). DNA samples were mixed with loading buffer (0.05% w/v bromophenol blue + 40% w/v sucrose + 0.1% w/v EDTA pH 8.0 and 0.5% w/v SDS) in 2:1 ratio. Eight  $\mu\text{l}$  aliquots of this solution were loaded in to the wells created by the comb. To identify size of the DNA segments, Lambda Hind III digested DNA marker (Sigma) was also loaded on each gel.

Electrophoresis was carried out by using Mini Sub<sup>TM</sup> or Wide DNA Cell (BIO-Rad). Electrophoresis was performed at 65 V for 2 hours. The gels were stained for 30 minutes in 10  $\mu\text{l}$ /100 ml of 1% ethidium bromide and photographed under UV light with Polaroid, positive/negative, instant 665 film. An extraction was thought successful if a single band, approximately 25 kilo base pairs (K bp) in size with no smearing was observed.



### 2.11.3. Determination of amount and purity of DNA

Quantification and purity of the DNA was based on the spectrophotometric measurement of the amount of ultraviolet irradiation absorbed by the nucleotide bases, as described by Sambrook *et al.* (1989). A DU®-65 Spectrophotometer (Beckmn Inst. UK. Ltd.) was used for taking the readings. Silica (Quartz); Ultra-Micro Spectrophotometer cuvet (Sigma) was used for the holding the samples and DNA hydration solution was used for calibrations of the spectrophotometer at required wavelengths.

Reading were taken for each DNA samples at wavelengths of 260 nm and 280 nm. The reading at 260 nm allowed calculation of the concentration of nucleic acid in the sample. An optical density (OD) of 1 corresponds to approximately 50 µg ml<sup>-1</sup> for double stranded DNA. The ratio between the reading at 260 nm and 280 nm (OD<sub>260</sub>/OD<sub>280</sub>) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have OD<sub>260</sub>/OD<sub>280</sub> value of 1.8. If there is contamination with protein or phenol, the ratio would be significantly less than the value given above.

### 2.11.4. DNA amplifications

For amplification of DNA the techniques of Williams *et al.* (1990) were adopted. Amplification reactions were performed in volumes of 50µl in UltraFlux™ PCR tubes (Scotlab). Reaction mixture consisted of 10 mM HCl pH 8.3, 50 mM KCl, 3 mM MgCl<sub>2</sub>, 200 µM each of dATP, dGTP, dCTP and dTTP, 1.5 units of AmpliTaq® DNA polymerase (Perkin Elmer, Cetus) 50 ng of genomic DNA and 0.3 µM of 10-mer primer (Operon Technologies, Alameda, California)

Amplification was carried out in GeneE (Techne) thermocycler, programmed for first cycle of 94°C for 2 min. (denaturation), 35 cycles of three steps each, i.e. 1 min. at 94°C (denaturation), 1 min. at 36°C (annealing) and 2 min. at 72°C (extension) followed by last cycle of 8 min at 72°C (for final extension).

After amplifications the reaction products were separated by electrophoresis with the same conditions used for the visualisation of DNA. DNA size marker used to check the fragment length of the PCR products were 123 DNA ladder or Lambda DNA EcoR I, Hind III digested (Sigma).

### 2.11.5. Genetic similarity estimate

Each of the clear RAPD bands across all the samples was assigned a number (1, 2, 3, .....n). Each band was treated as a unit character, and the samples were scored for the presence or absence of a band and coded as 1 or 0, respectively. Genetic similarity was calculated between the parent and regenerated plants/tissue cultured material, in pairs employing the equation (Nei and Li, 1979).

$$\text{Similarity} = (2 \times N_{ab}) / N_a + N_b$$

Where ' $N_{ab}$ ' is the number of common bands between sample 'a' and 'b', ' $N_a$ ' is the total number of bands scored for sample 'a' and ' $N_b$ ' is the number of total bands scored for sample 'b'. Because the similarity was estimated between different sample and the parent plant, therefore sample 'a' in all the cases was parent plant.

### 3. BASIC TECHNIQUES FOR TISSUE CULTURES OF *BRASSICA* SPECIES

#### 3.1. Introduction

Success or failure of any programme involving *in vitro* culture depends on some basic tissue culture techniques. These techniques may be specific to genotypes or species. Therefore establishment of basic culture techniques for each genotype is a prerequisite for any tissue culture programme. There are many factors which may affect a tissue culture programme positively or negatively. Decisions are mainly based on the choice of the worker, facilities available, needs of the project and the special requirements of the genotypes under study. Some of the factors which can effect a tissue culture system are mentioned here:-

**Explant.** Type, source, physical condition, age, preparation, size, sterilisation.

**Standard nutrient media.** Macro and micro nutrients, quantity and physical condition.

**Auxin and Cytokinin.** Type of auxin and cytokinin, concentrations of auxin and cytokinin for callus induction, subsequent callus cultures and regeneration.

**Culture containers.** Petri dishes, test tubes or flasks, size of the container, Plastic or glass container, numbers of explants per container.

**Culture conditions.** Light intensity, light period, temperature etc.

**Culture period.** Time taken by log phase.

For individual species and genotypes, all the factors mentioned above need to be investigated thoroughly by experimentation. However this may require a long time, and with the limitation of time available it was not possible to investigate all the variable factors under this project. Most decisions were made on the basis of facilities available and previous work of tissue culture on *Brassica* species. Only few factors which were considered essential were subjected to a more critical approach. The results of these experiments will be presented in this chapter.

The overall aim of this part of the project was to develop a suitable tissue culture system for the species of the *Brassica* under study. Emphasis was given mainly to two factors i.e. selection of suitable explant for callus production and plant regeneration and selection of optimum concentrations of auxin and cytokinin for callus induction, subsequent callus cultures, shoot regeneration and root formation.

Different researchers have used different auxins and cytokinins, depending on their choice or the specific need of the project. Some of the combinations of auxins and cytokinins used successfully for different studies involving *Brassica* species were, BAP (benzyl amino purine) + IAA (indole acetic acid, Kumar *et al.*, 1993); BAP+IBA (indole butyric acid) +GA3 (gibberellic acid, Chandler *et al.*, 1988a); BAP+NAA (naphthyleneacetic acid, Narasimhulu and Chopra, 1988); Kinetin+IAA (Jain *et al.*, 1986); Kinetin+NAA (Jain *et al.*, 1991; He and Cramer, 1993), and Zeatin+NAA (Narasimhulu *et al.*, 1993).

This review indicated that almost all types of commercially available auxins and cytokinin can be used successfully for *in vitro* cultures of *Brassica* species. The literature also showed that NAA and BAP are the most commonly used auxin and cytokinin. For this project, due to the limitation of time available it was not possible to study all growth regulators in different combinations, and then to decide which of them should be used. Therefore to save time it was decided that for each study at first only NAA and BAP would be investigated. If the results were satisfactory, then the same growth regulators would continued to be used, otherwise other growth regulators would also be investigated to select the appropriate ones.

### 3.2. Selection of explant type suitable for *in vitro* culture and regeneration

*In vitro* culture and regeneration in *Brassica* species has been achieved from many tissues and organs including leaf material (Lustinec and Horak, 1970; Dunwell, 1981), floral parts (Lizuka, *et al.*, 1978; Jin *et al.*, 1982), roots (Grant and Harney, 1982; Lazzeri and Dunwell, 1984 a,b) pollen and anthers (Keller, 1984; Dunwell and Cornish, 1985), cotyledons (Jain *et al.*, 1988) and hypocotyls (Kao *et al.*, 1990; Yang *et al.*, 1991; Narasimhulu *et al.*, 1993). Although different parts of *Brassica* plants can be used successfully as explant source in tissue cultures, each explant type has been shown to have a characteristic pattern of response which is specific to genotype. The kind of explant chosen can affect whether tissue culture can be successfully initiated and whether morphogenesis can be induced. Therefore selecting a suitable explant source before starting any tissue culture study is of great importance.

The first need of the overall research project was to select an appropriate explant source which could be used directly in experiments or for callus supply, throughout this research project. The explants need to be easy to handle, have good growth and regeneration capabilities in tissue culture and should be possible to supply in sufficient quantity, whenever needed. Therefore, this study was designed to investigate different explant sources and to select an appropriate one for this project.

As already described almost all parts of the *Brassica* plants can be used as explant source for tissue culture, but for this study only four of them were chosen for investigation. Explant sources selected for investigation were, leaf discs with vein, leaf discs without main vein, petioles and hypocotyls. The reason for selecting these explant sources was that less time is required to get appropriate age of explant, immediate supply in sufficient quantity was possible and sterilisation and handling was easy. The aim of this study was to check the response of these explants for callus initiation and shoot regeneration and to select an explant capable of callus initiation and, especially, shoot regeneration.

#### 3.2.1. Experimental procedure

All the explants were sterilised and prepared as described under Section 2.4. Sixteen types of culture media, differing only in their concentrations of growth regulators (NAA/BAP), were studied in this experiment. Levels of NAA and BAP investigated were 0, 0.1, 1, and 10 mg l<sup>-1</sup> of each in all possible combinations. The combinations of the growth regulators is shown in Table 3.1. Each type of medium

was prepared (Section 2.3) separately and dispensed (approximately 15 ml) into petri dishes of 50 mm diameter.

**Table 3.1. Combinations of NAA/BAP added to culture media to study the effect of growth regulators on *in vitro* cultures. All figures are in mg l<sup>-1</sup>.**

NAA BAP	0	0.1	1.0	10
0	0/0	0.1/0	1/0	10/0
0.1	0/0.1	0.1/0.1	1/0.1	10/0.1
1.0	0/1	0.1/1	1/1	10/1
10	0/10	0.1/10	1/10	10/10

Explants were excised, placed on the surface of the media and pressed gently to make proper contact with the media. Three pieces of explants were cultured in each petri dish, and each petri dish was considered as a single treatment. A total of 5 replications were used for each treatment and all the petri dishes were randomised in the culture room. Cultures were observed weekly and final observations were collected after 4 weeks of incubation. Because the aim of this investigation was to study the capabilities of explants for callus induction and shoot regeneration, the data collected was mainly based on the quality rather than quantity. From the same experiment categorical data was collected for two variables i.e. callus initiation and shoot formation. Data was subjected to statistical analysis using the  $\chi^2$ -test, separately for each variable and each factor.

### 3.2.2. Callus initiation

Ideas for scoring the callus growth were taken from the studies of Dietert *et al.* (1982) and Ogihara and Tsunewaki (1979). In the both studies scoring was based on the area covered by the callus produced. In this study different explants types studied were different in shapes and sizes. It was very difficult to compare the callus by the area covered. Therefore, while scoring the callus, emphasis was given to the actual quantity of callus induced in all directions. The scoring was made by visual estimation. The classes formulated to categorise the explants are as follows.

**Category 1:-** No callus initiation

**Category 2:-** Slight callus induction, when there was only a thin layer of callus at the cut ends of the explants (approximately <100 mg on each explant piece).

**Category 3:-** Good callus initiation, if the callus induced by an explant was enough for subculture as one callus piece (approximately  $\geq 100$  mg) then it was graded as 3.

Each explant piece was individually observed and put under one of the groups mentioned above. Frequencies of explants under each group were counted and the data was subjected to statistical analysis.

All types of the explants, under study, showed callus induction on most of the media studied. Usually callusing was observed at the cut ends of explants. As shown in the Table 3.2 callus induction capability was very much affected by growth regulator concentrations as well as their combinations.

**Table 3.2. Callus induction on different explant sources.**

Genotypes →		Curly Kale				<i>Brassica rapa (R.C.)</i>				Winter Oilseed Rape			
Explant source → NAA      BAP mg l <sup>-1</sup>		LD	LD/V	P	H	LD	LD/V	P	H	LD	LD/V	P	H
0	0	-	-	-	-	-	-	-	-	-	-	-	-
0	0.1	+	+	+	+	+	+	+	+	+	+	+	+
0	1	+	+	+	+	+	+	+	+	+	+	+	+
0	10	+	+	+	+	+	+	+	+	+	+	+	+
0.1	0	+	+	+	+	+	+	+	+	+	+	+	+
0.1	0.1	++	++	++	++	++	++	++	++	++	++	++	++
0.1	1	+	++	++	+	++	++	++	+	+	+	++	++
0.1	10	+	+	+	+	+	+	+	+	+	+	+	+
1	0	+	+	+	+	+	+	+	+	+	+	+	+
1	0.1	++	++	++	++	++	++	++	++	++	++	++	++
1	1	++	++	++	++	++	++	++	++	++	++	++	++
1	10	+	+	+	+	+	+	+	+	+	+	+	+
10	0	+	+	+	+	+	+	+	+	+	+	+	+
10	0.1	+	+	+	+	+	+	+	+	+	+	+	+
10	1	+	+	++	+	++	+	++	+	+	+	++	++
10	10	++	+	++	++	++	+	++	+	+	++	+	++

**Explant sources:**

LD/V :- Leaf disks with vein  
LD :- Leaf disks without vein  
P :- Petioles  
H :- Hypocotyls

**Callus growth**

"-" No callus induction  
"+" Slight callus induction  
"++" Good callus induction

Results of  $\chi^2$ -test analysis has been shown in Table 3.3.  $\chi^2$ -testing indicated that there were no significant differences between different explant types under study for their ability to induce callus, regardless of the yield of the callus. Only species in

general, regardless of the explant source showed significant difference ( $P=0.04$ ) for callus induction capabilities.

**Table 3.3. Results of different  $\chi^2$ -tests for callus induction from four different explant sources of 3 *Brassica* species.**

Factor	Total No. of explants	D.F.	Chi- square	Significance ( $P$ )
Explants of Curly Kale	240	3	2.582	0.859
Explants of Winter Oilseed Rape	240	3	4.423	0.374
Explants of <i>Brassica rapa</i> (R.C.)	240	3	6.458	0.619
Explants (in general)	720	3	3.759	0.709
Genotypes	960	2	9.844	0.040

From these observations it was decided that callus formation would present no difficulties for any of the explants under study unless there was any problem of regeneration. Therefore the decision as to which explant to use, was left to the successful regeneration from explants.



### 3.2.3. Shoot regeneration

For shoot formation each explant piece was marked separately for one of the two categories i.e. **Yes** or **No**. Explants showing at least shoot bud formation or more regeneration were marked as 'Yes', while the other explants showing no signs of shoot differentiation were marked as 'No'. Frequency of explant within each group was counted and subjected to statistical analysis.

**Table 3.4. Shoot initiation on different explant sources.**

Genotypes →		Curly Kale				<i>Brassica rapa</i> (RC)				Winter Oilseed Rape			
Explant source → NAA      BAP mg l <sup>-1</sup>		LD	LD/V	P	H	LD	LD/V	P	H	LD	LD/V	P	H
0	0	-	-	-	-	-	-	-	-	-	-	-	-
0	0.1	-	-	-	+	-	-	-	-	-	-	-	-
0	1	-	-	+	+	-	-	-	-	-	-	-	+
0	10	-	-	+	+	-	-	-	-	-	-	+	+
0.1	0	-	-	-	+	-	-	-	-	-	-	-	-
0.1	0.1	-	-	-	+	-	-	-	-	-	-	-	-
0.1	1	-	-	-	+	-	-	-	-	-	-	-	-
0.1	10	-	-	+	+	-	-	-	-	-	-	+	+
1	0	-	-	-	-	-	-	-	-	-	-	-	-
1	0.1	-	-	-	-	-	-	-	-	-	-	-	-
1	1	-	-	-	+	-	-	-	-	-	-	-	-
1	10	-	-	+	+	-	-	-	-	-	-	-	+
10	0	-	-	-	-	-	-	-	-	-	-	-	-
10	0.1	-	-	-	-	-	-	-	-	-	-	-	-
10	1	-	-	-	-	-	-	-	-	-	-	-	-
10	10	-	-	-	+	-	-	-	-	-	-	-	-

**Explant sources:**

LD/V :- Leaf discs with vein  
 LD :- Leaf discs without vein  
 P :- Petioles  
 H :- Hypocotyls

**Shoot regeneration**

"+" :- yes  
 "-" :- No

Shoot regeneration by different types of explant sources grown on 16 types of media differing only in their growth regulator concentrations and combinations is shown in Table 3.1. Shoot regeneration was very much critical to media, explant source and genotypes (Table 3.4). Results of the  $\chi^2$ -tests are presented in Table 3.5. In the case of Curly kale such  $\chi^2$ -test showed highly significant differences for the shoot regeneration ability of different explants. Maximum shoots differentiation was observed when hypocotyls were used as explant (Fig 3.1 a). Petioles showed little shoot formation while leaf discs showed no shoots formation. With winter oilseed rape, although the  $\chi^2$ -test of shoot regeneration showed highly significant differences

among the explant sources, very little shoot differentiation was observed. Again maximum shoot formation was observed on hypocotyl explants (Fig 3.1 b). *Brassica rapa* showed no differentiation on any of the explants under study therefore  $\chi^2$ -test showed no significant differences among the explant types (table 3.5, Fig 3.1 c).

**Table 3.5. Results of different  $\chi^2$ -tests for direct shoot regeneration from 4 different explant sources of 3 *Brassica* species.**

Factor	Total No. of explants	D.F.	Chi-square values
Explants of Curly Kale	240	3	220.093 **
Explants of Winter Oilseed Rape	240	3	35.607 **
Explants of <i>Brassica rapa</i> (R.C.)	240	3	----- N.S.
Explants (in general)	720	3	242.502 **
Genotypes	960	2	147.327 **

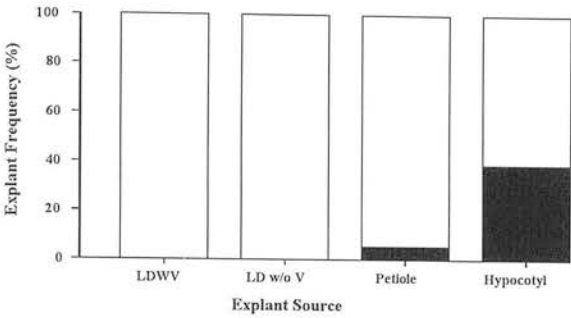
N.S. Non-significant ( $P \geq 0.05$ )

\*\* Highly significant ( $P < 0.01$ )

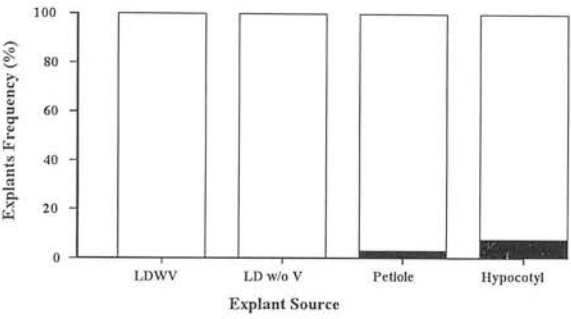
When the genotypes were compared for shoot regeneration  $\chi^2$ -test showed highly significant differences (Table 3.5). In general maximum shoot regeneration was on the explants of curly kale. Little shoot formation was observed in case of winter oilseed rape and not at all with *Brassica rapa* (Fig 3.2a).

When differences in explants were compared with each other, regardless of species and growth regulator concentrations,  $\chi^2$ -test showed highly significant differences among the explant type (Table 3.5). The maximum shoot differentiation was when hypocotyls were used as explant source material (Fig 3.2 b). From these results it is clear that hypocotyl explants showed maximum shoot differentiation. Therefore hypocotyls were preferred over other explant sources studied and it was decided to use the hypocotyl explant in this project.

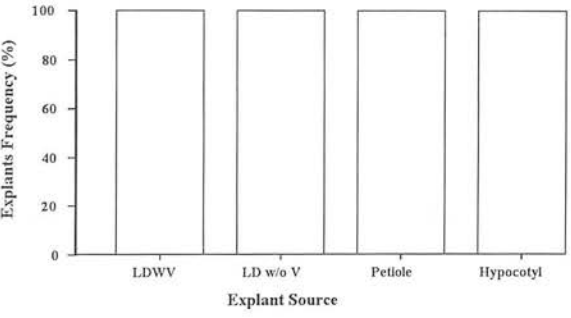
(a)



(b)



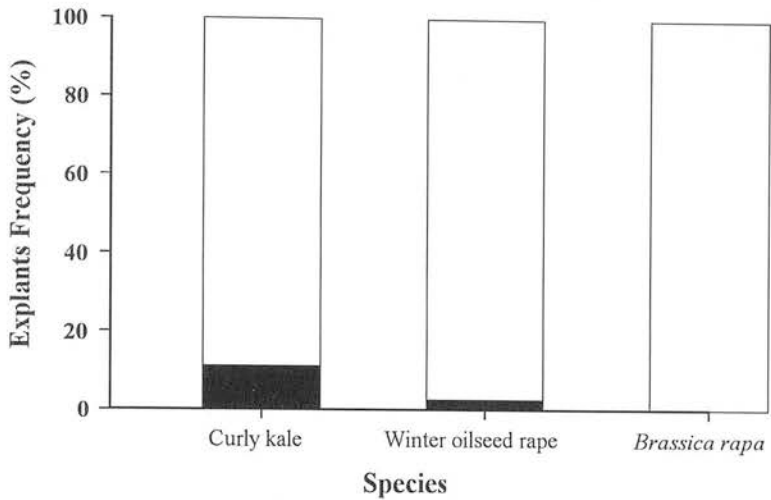
(c)



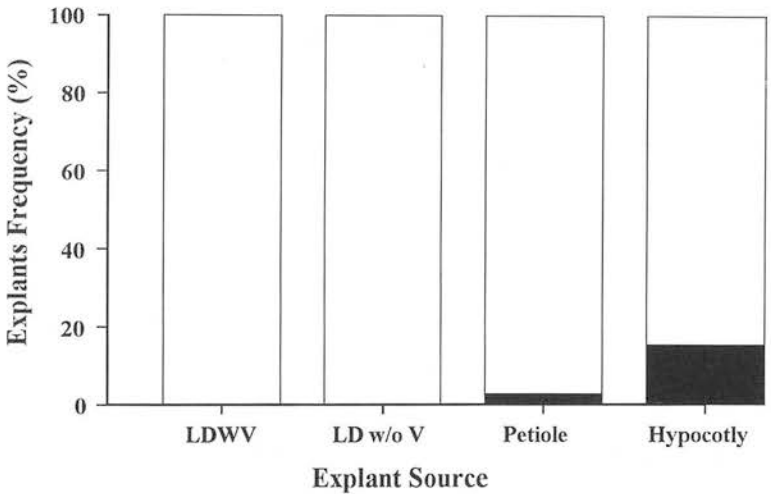
■ Shoot Differentiation    □ No Differentiation

Fig. 3.1. Influence of explant type on shoot differentiation frequency from different *Brassica* species. Frequencies of explants showing differentiation was collected after 4 weeks of incubation on MS-media supplemented with NAA and BAP in different combinations, with 5 replication and 3 explant pieces in each replication. (a) Curly kale, (b) Winter oilseed rape, and (c) *Brassica rapa*.  
LDWV⇒ leaf discs with vein  
LD w/o V⇒ leaf discs without vein.

(a)



(b)



■ Shoot Differentiation    □ No Differentiation

**Fig. 3.2.** Influence of genotypes and explant type on shoot differentiation frequency. Shoot formation was investigated on 16 different combinations of NAA and BAP, with 5 replication and 3 explant pieces in each replication. Frequencies of explants showing differentiation, (a) from different genotypes regardless of explant type, (b) from different explant sources regardless of genotypes. LDWV⇒ leaf discs with vein and LD w/o V⇒ leaf discs without vein.

### 3.3. Optimisation of NAA and BAP for callus production.

For successful callus yield from *in vitro* cultures, the concentrations of both auxin and cytokinin is critical. Many workers have already reported optimisation of growth regulators for callus production in different studies (Dietert *et al.*, 1982; Murata and Ortan, 1987; Das, 1991). Therefore the next stage of the project was the optimisation of the levels of growth regulators for callus induction and subsequent callus cultures of the different *Brassica* species, under study. As already discussed in Section 3.1, many types of auxin and cytokinin can be used for *in vitro* cultures, but due to limitation of time available it was not possible to study the effect of all the growth regulators. From the literature studied and results of previous studies (Section 3.2.2) it is clear that NAA and BAP in combination gave satisfactory results for callus production from different *Brassica* species. Therefore it was decided that NAA and BAP would be used to study appropriate concentration.

Form the literature available it is clear that the concentrations of NAA and BAP mostly used in different studies ranged from 0.01-5.0 mg l<sup>-1</sup>. But to provide a more comprehensive view it was decided that the concentrations of NAA and BAP from 0 to 10 mg l<sup>-1</sup>, increasing with logarithmic scale, would be tested to investigate a narrow range. Later on from that range the optimum concentration for callus induction and subsequent callus cultures would be finalised.

It has been demonstrated by many researchers that the initial content of the endogenous growth regulators may effect the requirement of auxin and cytokinin for *in vitro* cultures. Thus the level of endogenous auxin/cytokinin in hypocotyls may differ from that of the callus, isolated from primary explant source. Therefore it is necessary to identify the appropriate hormone balance for both initiation of callus and for subsequent callus growth. Thus the study was divided into needs for initiation and needs for subsequent and continuing culture of callus.

#### 3.3.1. Experimental procedure

To find out a suitable range of growth regulators for callus initiation, the hypocotyls were cultured on MS-media supplemented with different concentrations of NAA and BAP, ranging from 0 to 10 mg l<sup>-1</sup> each. The combinations of the growth regulators studied was as shown in Table 3.1. On the basis of the results obtained from this experiment, a narrow range was selected and the experiment was effectively repeated to identify the optimum concentration of NAA and BAP for callus initiation and subsequent sub-culture.

Procedures for the sowing of seed to take the hypocotyls and the preparation of explants are as described in general material and methods (Section 2.4). About 10 mm segments of hypocotyls were cut and mixed together. Three pieces of hypocotyls were picked randomly and cultured in each petri dish of 50 mm diameter, on all the media types.

To identify the optimum concentrations of hormones for continuing callus cultures, primary callus (first generation callus derived from the hypocotyl explant) was used as the appropriate starting material. Callus grown on hypocotyl explants was harvested carefully to ensure no original explant material was included. This was then cut into small pieces ( $\approx 100$  mg) and placed on culture media. Three callus pieces were placed in each petri dish of 50 mm diameter. All the petri dishes were sealed with double layer of parafilm and placed in culture room. The experimental design used was CRD, with five replications.

Because the concentration of growth regulators for callus production is specific to genotype/species (Dietert *et al.*, 1982; Das, 1991), therefore it was needed to optimise the concentration of NAA and BAP separately for each of the species under study. Thus the experiments were repeated for each species.

Callus fresh weight from hypocotyl segments and relative fresh weight gain from primary callus was calculated (Section 2.7) after an incubation period of 4 weeks. The data was subjected to statistical analysis, through Fisher (1958) standard analysis of variance techniques (Steel and Torrie, 1980) to obtain the level of significance among different treatments.

### **3.3.2. Cultures of curly kale**

#### **3.3.2.1. Effect of NAA and BAP on callus induction from hypocotyl explants of curly kale (a).**

The aim of this experiment was to find out a suitable range of NAA and BAP for callus induction, using hypocotyl segments as explant. Sixteen different media tested in this experiment differed only in their growth regulator concentrations (Table 3.1). The experimental design used was CRD 2-factors-factorial. Analysis of variance (ANOVA) revealed highly significant differences among the levels of treatments, individually as well as in interaction (Appendix 3.1). Fig 3.3 (a) shows that both NAA and BAP are necessary for good callus initiation and growth. There

was no callus initiation at all when the concentration of NAA and BAP both were zero. Maximum callus induction, observed in this experiment, was on the medium with  $1 \text{ mg l}^{-1}$  of each NAA and BAP, which was significantly more than that on other combinations (LSD (Least Significant Differences)=0.282,  $P=0.05$ ).

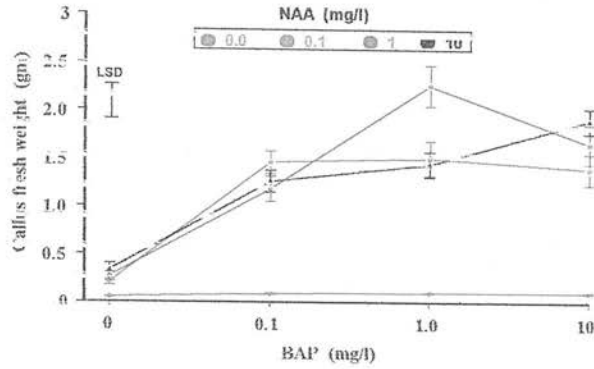
In general the results indicated that there was a good callus growth when the NAA and BAP were in 1:1 ratio or the concentration of NAA was slightly more than that of the BAP (Fig 3.3a).

Since the levels investigated in this experiment were of the order of  $10\times$  differences between levels of each hormone, it was decided to investigate further to refine hormones needs. For this purpose, levels around the optimum levels, found in this experiment (i.e.  $1 \text{ mg l}^{-1}$  each of NAA and BAP), were investigated further.

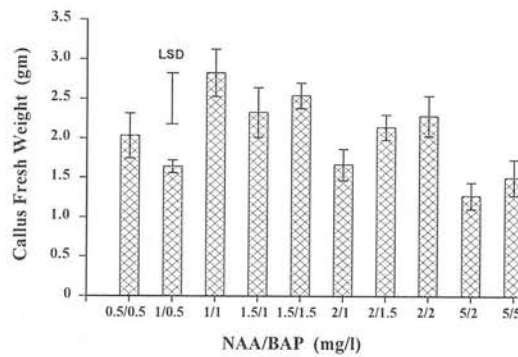
#### **3.3.2.2. Effect of NAA and BAP on callus induction from hypocotyl explants of curly kale (b).**

The aim of this experiment was to identify the optimum concentrations of NAA and BAP for callus initiation using hypocotyls as explant source. New combinations and levels of NAA and BAP were chosen on the basis of the results of the previous experiment (Section 3.3.2.1). The new levels investigated were ranged from 0.5 to  $5.0 \text{ mg l}^{-1}$  in specific combinations of both hormones. A total of 10 different combinations were used, in which NAA and BAP were either in 1:1 ratio, or concentration of the NAA was kept slightly higher than that of BAP (Table 3.6). Each combination was considered as a single treatment. After 4 weeks of incubation callus fresh weight from each petri dish was calculated and the data was analysed using standard analysis of variance technique.

(a)



(b)



(c)

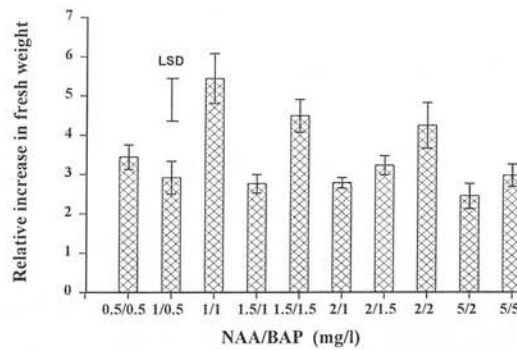


Fig. 3.3. Influence of different levels of NAA and BAP on callus induction and subsequent callus culture of Curly kale. Data was collected after 4 weeks of incubation. Mean $\pm$ SEM, n=5.

(a)  $\Rightarrow$  Individual effect of NAA and BAP on callus initiation, using hypocotyl segments.

(b)  $\Rightarrow$  Effect of NAA and BAP, in combination, on callus initiation.

(c)  $\Rightarrow$  Effect of NAA and BAP on subsequent callus cultures.



**Table 3.6. Combination of NAA/BAP added to the tissue culture media to study the effect of growth regulators on *in vitro* cultures. All figures are in mg l<sup>-1</sup>.**

NAA BAP	0.5	1	1.5	2	5
0.5	0.5/0.5	1/0.5	-	-	-
1	-	1/0.1	1.5/1	2/1	-
1.5	-	-	1.5/1.5	2/1.5	-
2	-	-	-	2/2	5/2
5	-	-	-	-	5/5

Analysis of variance revealed highly significant differences among the treatments (Appendix 3.2). Again maximum callus growth was observed on the medium with 1/1 mg l<sup>-1</sup> of NAA and BAP (Fig 3.2 b). Callus initiation/growth at this media was not significantly more than that on the media with 1.5/1 and 1.5/1.5 of NAA and BAP, but significantly more than that on all other media type (LSD=0.65,  $P=0.05$ ). No significant differences for the callus initiation on these three concentrations revealed that the concentration of NAA and BAP from 1-1.5 mg l<sup>-1</sup> each, in 1:1 or NAA slightly higher than BAP is optimum for callus induction from hypocotyls of curly kale. To make a standard for the curly kale 1 mg l<sup>-1</sup> of each NAA and BAP will be used for callus initiation.

### 3.3.2.3. Effect of NAA and BAP on callus maintenance of curly kale.

From the previous two experiments optimum concentrations for callus induction using hypocotyl segments as explant was determined. The aim of this experiment was to confirm whether these levels of NAA and BAP for callus induction were also optimum concentration for subsequent callus cultures.

Levels of NAA and BAP investigated in this experiment were the same as in the previous experiment (Table 3.6). Primary callus produced from hypocotyl segments was used as starting material. After an incubation period of 4 weeks, final fresh weight of callus was collected. Relative fresh weight gain was calculated (Section 2.7) and the data was analysed using ANOVA.

Analysis of variance showed highly significant difference between treatments (Appendix 3.3). The results achieved were almost the same as when the hypocotyls



were used as explant source material (Section 3.3.2.2). Although the highest relative fresh weight gain was observed on the medium supplemented with 1 mg l<sup>-1</sup> each of NAA and BAP, the callus growth was not significantly more as compared with that on the media with 1.5/1 and 1.5/1.5 of NAA and BAP mg l<sup>-1</sup>. The callus growth at these media were significantly more than that on all other media type (LSD=1.113,  $P=0.05$ ; Fig 3.3 c).

No significant differences among the callus growth at the concentrations of 1/1, 1.5/1 and 1.5/1.5 mg l<sup>-1</sup> of NAA and BAP indicated that any one of these combinations could be used for a good callus growth. But to give the same treatment to each culture a single combination was standardised. Therefore it has been decided that 1 mg l<sup>-1</sup> of NAA and BAP would be used for callus induction and subsequent cultures of curly kale, throughout this project.

### **3.3.3. Culture of winter oilseed rape**

To optimise the levels of NAA and BAP for callus induction and subsequent cultures of winter oilseed rape, all the studies described under Section 3.3.2. were repeated for winter oilseed rape.

#### **3.3.3.1. Effect of NAA and BAP on callus induction from hypocotyl explants of winter oilseed rape (a).**

Callus fresh weight from each petri dish, on 16 types of culture media (Table 3.1) tested, was measured and analysed statistically. Statistical analysis revealed highly significant differences among the levels of NAA and BAP individually as well as in interaction (Appendix 3.4). Both auxin and cytokinin were necessary for callus induction (Fig 3.4a). In general when ever the NAA and BAP were in balance (1:1 ratio) there was a good callus growth. Maximum average callus growth was observed on 1/1 mg l<sup>-1</sup> of NAA/BAP. Next highest averages were observed on the media supplemented with 10/10 of NAA/BAP mg l<sup>-1</sup>. Further comparison of mean callus yields at different hormones levels showed no significant differences at these two levels, which were significantly more than that on all other media types (LSD 0.341,  $P=0.05$ ). These results indicate that optimum concentration for callus induction in case of winter oilseed rape lies with in the range from 1-10 mg l<sup>-1</sup> of NAA and BAP, with auxin and cytokinin approximately in balance.

#### **3.3.3.2. Effect of NAA and BAP on callus induction from hypocotyl explants of winter oilseed rape (b).**

To find out the optimum levels of NAA and BAP the previous experiment (3.3.3.1) was repeated with new combination of concentration. New range, levels

and combinations were selected on the basis of the results of the previous experiment. New concentrations studied were ranging from 1-10 mg l<sup>-1</sup> of NAA and BAP and the combinations studied has been shown in Table 3.7. Each combination studied was considered as a single treatment.

**Table 3.7. Combination of NAA/BAP added to the tissue culture media to study the effect of growth regulators on *in vitro* cultures. All figures are in mg l<sup>-1</sup>.**

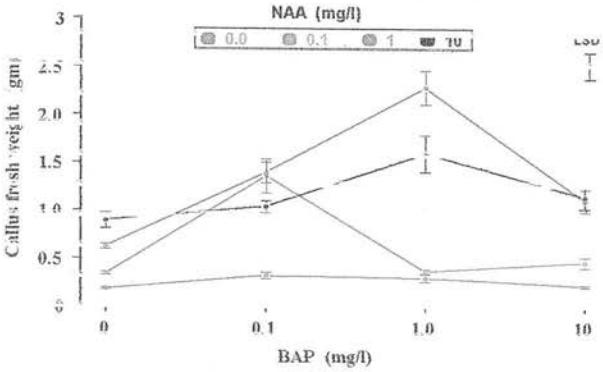
NAA BAP	1	2	5	10
1	1/1	2/1	5/1	-
2	-	2/2	5/2	10/2
5	-	-	5/5	10/5
10	-	-	-	10/10

Analysis of variance showed highly significant differences among the average callus fresh weight on different treatments (Appendix 3.5). Generally hypocotyl segments on the media with balance NAA/BAP showed better callus induction than that on other culture media. Maximum average callus growth was observed when NAA and BAP both were used at the rate of 2 mg l<sup>-1</sup> (Fig 3.4b) and the callus growth at this concentration was significantly more than that on all other combinations tested in this experiment (LSD=0.63, *P*=0.05). Thus it can concluded that 2 mg l<sup>-1</sup> of NAA and BAP could be used successfully to obtain a good callus yield from hypocotyl segments of winter oilseed rape.

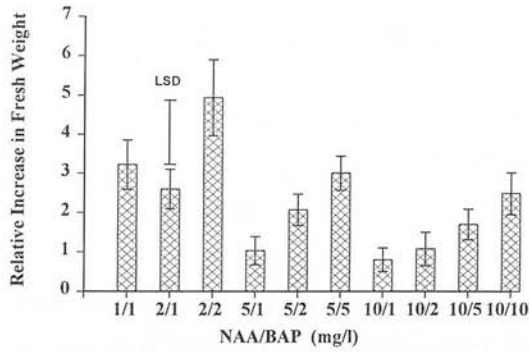
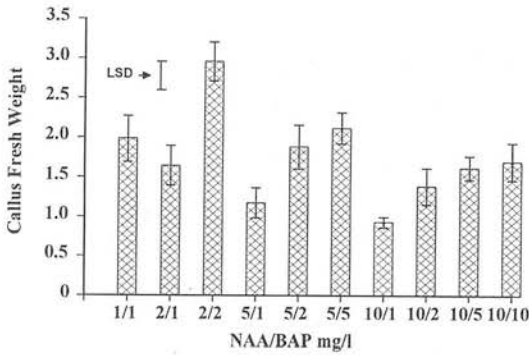
### 3.3.3.3. Effect of NAA and BAP on callus maintenance of winter oilseed rape.

This study was carried out to finalise the concentrations of NAA and BAP for callus cultures of Winter oilseed rape using primary callus as source material. Combinations of concentrations used were the same as in case of previous experiment (Table 3.7).

(a)



(b)



**Fig. 3.4.** Effect of different concentrations of NAA and BAP on callus culture of Winter oilseed rape. Data collected after 4 weeks of incubation. Mean±SEM, n=5.

(a)⇒ Individual effect of NAA and BAP on callus initiation, using hypocotyl segments.

(b)⇒ Effect of NAA and BAP, in combination, on callus initiation.

(c)⇒ Effect of NAA and BAP on subsequent callus cultures.

The results achieved were almost the same as when the hypocotyls were used as explant source. Analysis of variance indicated highly significant differences for average callus yield among different combinations (Appendix 3.6). Combination with 2 mg l<sup>-1</sup> each of NAA and BAP showed significantly more callus yield over that on all other combinations (LSD=1.506,  $P=0.05$ , Fig 3.4c). Conclusions drawn from these studies were that 2 mg l<sup>-1</sup> of NAA and BAP are suitable for a good callus growth in case of Winter oilseed rape.

### 3.3.4. Culture of *Brassica rapa* (R.C.)

The same studies describe under Section 3.3.2. were again repeated to optimise the growth regulator concentration for callus induction and subsequent callus cultures of *Brassica rapa*.

#### 3.3.4.1. Effect of NAA and BAP on callus induction from hypocotyl explants of *Brassica rapa* (a).

Callus fresh weight produced by hypocotyl segments of *Brassica rapa* on 16 different media (Table 3.1) was measured and subjected to statistical analysis. Analysis of variance revealed highly significant differences among the treatments individually as well as for interaction (Appendix 3.7). Maximum callus growth was observed at the medium with 1 mg l<sup>-1</sup> of NAA and BAP each. Mean comparison using LSD (0.113,  $P=0.05$ ) values, showed significant differences in average callus fresh weight on the medium with 1 mg l<sup>-1</sup> of each of NAA and BAP and that of other media (Fig 3.5a).

#### 3.3.4.2. Effect of NAA and BAP on callus induction from hypocotyl explants of *Brassica rapa* (b).

To finalise the optimum concentration, for callus induction on hypocotyl of *Brassica rapa*, the new combinations and the levels of NAA and BAP were the same as used in the Section 3.3.2.2 (Table 3.6).

Analysis of variance showed highly significant differences among the treatments (Appendix 3.6). Maximum callus induction was observed on the medium with 1 mg l<sup>-1</sup> of NAA and BAP. Callus growth at the medium with 1 mg l<sup>-1</sup> was significantly more as compared with that on all other media type (LSD=0.404,  $\alpha=0.05$ ). The results also indicated that in general, callus growth was more in the media with an equal balance (1:1) of auxin and cytokinin as compared with that on media with other proportions of growth regulators concentrations.

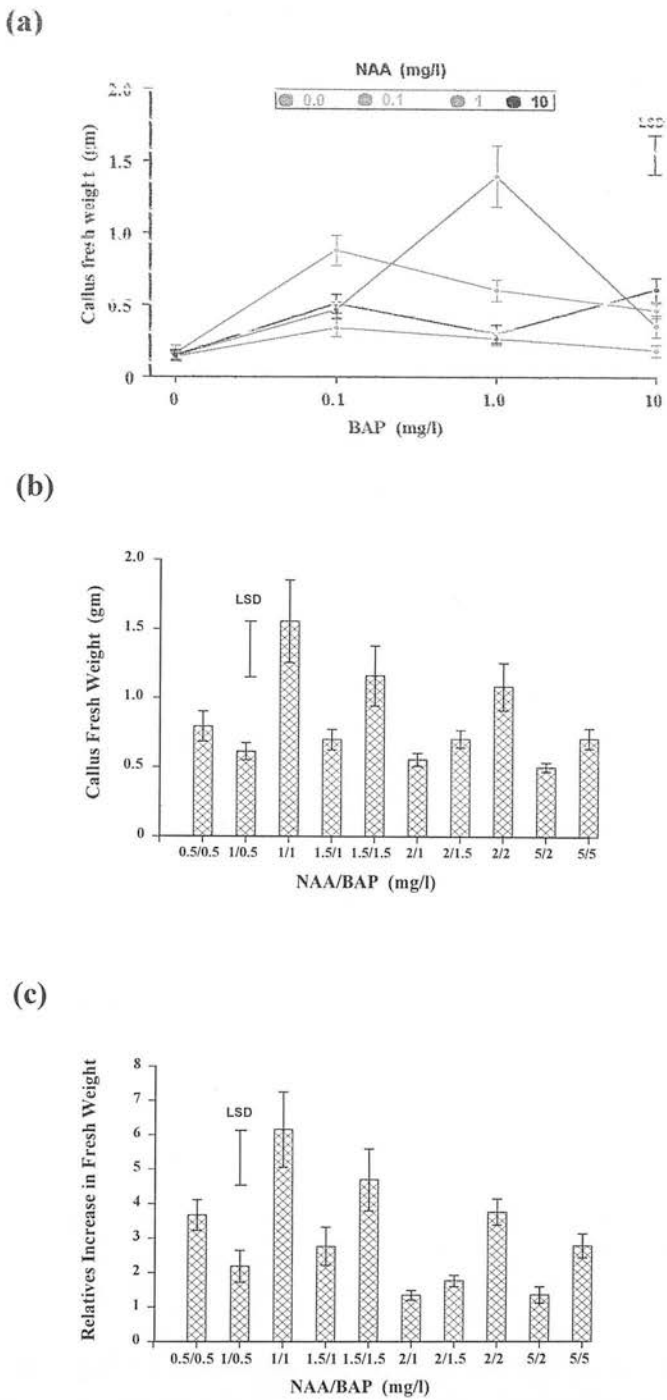
Theses results indicate that 1-1.5 mg l<sup>-1</sup> each of NAA and BAP, in balance, gives the best callus induction from hypocotyl segments of *Brassica rapa*. To standardise a single combination, it was decided that 1 mg l<sup>-1</sup> each of NAA and BAP would be used for callus induction in case of *Brassica rapa*.

#### 3.3.4.3. Effect of NAA and BAP on callus maintenance of *Brassica rapa*.

To finalise the optimum concentration of the growth regulators for subsequent callus cultures of *Brassica rapa* the previous study was repeated with the same growth regulators combinations. Primary callus, taken from the hypocotyls was used as starting material.

Analysis of variance (Appendix 3.9) showed highly significant differences for callus growth at different growth regulator concentrations. Maximum callus growth was observed on the medium with 1 mg l<sup>-1</sup> of NAA and BAP. Comparison of mean callus growth at different media type (LSD=1.59,  $P=0.05$ ) showed that callus growth at the medium with 1/1 mg l<sup>-1</sup> NAA/BAP was significantly more than that on all other media except that with 1.5/1.5 mg l<sup>-1</sup> NAA/BAP. The results indicated that, under the circumstances of these studies, 1-1.5 mg l<sup>-1</sup> of NAA and BAP in balance shows maximum callus growth. Again, to standardise a single combination it was decided that tissue culture media would be supplemented with 1 mg l<sup>-1</sup> of NAA and BAP for callus induction as well as for subsequent callus cultures of *Brassica rapa*.

After establishing a successful protocol for callus cultures, work was carried out to establish a suspension culture. This would allow comparison of selection efficiency from different culture system (callus culture, suspension culture and plated cells). many attempts were made to establish suspension culture and plated cell culture of each of the species under investigation (Appendix 3.1). None were successful. Therefore only callus culture could be used for *in vitro* selection in this project.



**Fig. 3.5.** Influence of different levels of NAA and BAP on callus induction and subsequent callus culture of *Brassica rapa*. Data collected after 4 weeks of incubation. Mean±SEM, n=5.

(a)⇒ Individual effect of NAA and BAP on callus initiation, using hypocotyl segments.

(b)⇒ Effect of NAA and BAP, in combination, on callus initiation.

(c)⇒ Effect of NAA and BAP on subsequent callus culture.

### 3.4. Optimisation of NAA and BAP for shoot regeneration.

Following the optimisation of the levels of growth regulators for callus cultures, the next requirement of the project was the optimisation of the levels of auxin and cytokinin for shoot regeneration for the different *Brassica* species under study. Several reports indicated the optimisation of growth regulators for *in vitro* regeneration in *Brassica* species. Yang *et al.* (1991) studied the effect of BAP (1, 2, 3, 4, 5 or 10 mg l<sup>-1</sup>) in combination with NAA (0.1, 0.2, 0.5, 1 mg l<sup>-1</sup>) and the best regeneration was found on the medium with high BAP with NAA ≤ 0.1 mg l<sup>-1</sup>.

From the experiments, described under Section 3.2.3, it was also observed that shoot formation was generally successful when the culture medium was supplemented with BAP only or in combination with a small quantity of NAA. On the basis of the literature available and the previous studies, a programme of experiments was carried out to investigate the appropriate levels of NAA/BAP for shoot regeneration. The concentrations of growth regulators and their combinations investigated are shown in Table 3.8.

**Table 3.8. Combination of NAA/BAP added to the tissue culture media to study the effect of growth regulators on shoot regeneration from the callus of different *Brassica* species. All figures are in mg l<sup>-1</sup>.**

<div>NAA BAP</div>	0	0.1	1
0.5	-	0.1/0.5	-
1	0/1	0.1/1	-
2	0/2	0.1/2	1/2
5	0/5	0.1/5	1/5

While studying the regeneration in tissue culture it was not possible to measure the regeneration quantitatively. Therefore the methodology of Ogihara and Tsunewaki (1979) was adopted to measure the shoot regeneration from different explants at different growth regulator levels. Slight changes were made in this method. Ogihara and Tsunewaki (1979) graded the callus pieces into three classes i.e. no differentiation, shoot primordia formation and shoot formation. In tissue culture, some times shoot formation occurs which may not develop into normal shoots. Therefore in this study the last grade was divided in to two classes, to



differentiate between the growth of the regenerated shoot i.e. shoots <1 cm and the shoots  $\geq 1$  cm.

### 3.4.1. Experimental procedure

Callus was produced using hypocotyl explants of different *Brassica* species, at the optimum growth regulator concentrations specific to the species. One month old callus was harvested and cut into small pieces ( $\approx 100$  mg each). Callus pieces were pooled together. Culture media with different concentrations of NAA and BAP (Table 3.8) was prepared and dispensed into 60 ml specimen container. Three pieces of callus were picked randomly and placed on the medium in each container. Each combination of the growth regulators was considered a single treatment, and there were a total of 5 replications for each treatment. All the containers were randomised in the culture room.

After 4 weeks of incubation categorical data was collected. Callus pieces were graded in to the following 4 classes.

1. No visible differentiation
2. Only shoot bud formation
3. Shoots < 1 cm
4. Shoot  $\geq 1$  cm

Number of explants with in each group was determined. Data was subjected to  $\chi^2$ -test to investigate the statistical differences for regeneration ability for the different types of media under study. Results of the  $\chi^2$ -tests are shown in table 3.9.

**Table 3.9. Results of different  $\chi^2$ -tests for shoot regeneration on different concentrations of NAA in combination with BAP.**

Factor	D.F.	Chi-square
Primary callus of Curly Kale	27	47.87 **
Primary callus of Winter Oilseed Rape	27	----- N.S.
Primary callus of <i>Brassica rapa</i> (R.C.)	27	----- N.S.
** Highly significant ( $P < 0.01$ )	N.S.	Non-significant at $P \geq 0.05$

### 3.4.2. Shoot regeneration in curly kale

$\chi^2$ -test (Table 3.9) of shoot regeneration ability, showed highly significant differences for shoot regeneration frequencies at different NAA/BAP concentrations. Considering the categories 3 and 4 as successful shoot formation, Fig 3.6 shows that maximum shoot formation was observed on the media with 1 mg l<sup>-1</sup> of BAP only. At this concentration about 24% of the explant pieces showed  $\geq 1$  cm long (grade 4) regenerated shoots. About 60% of total explant pieces showed shoot regeneration (grade 3 and 4). Less than 20% of total explant pieces showed no differentiation. The frequency of the explant pieces showing shoot on any other media was not more than 40%. Therefore it was decided that MS-media supplemented with 1 mg l<sup>-1</sup> of BAP only would be used as regeneration media for curly kale cultures.

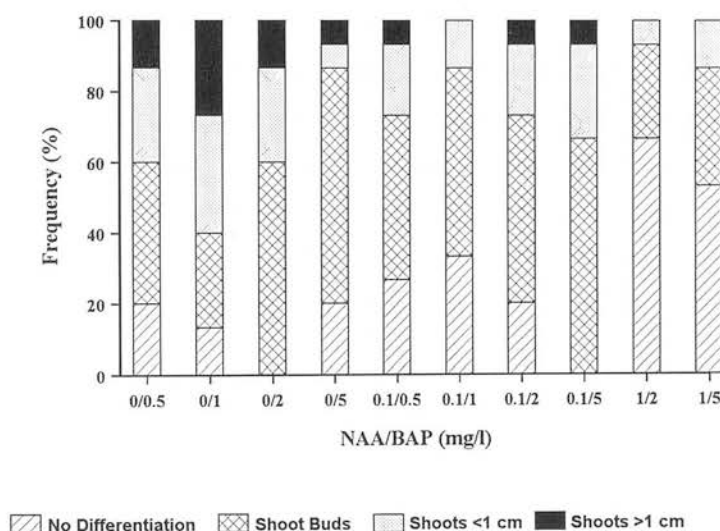


Fig. 3.6. Shoot regeneration frequency on different levels of NAA in combination with BAP. Source material primary callus of Curly Kale. Mean $\pm$ SEM, n=5.

### 3.4.3. Shoot regeneration in winter oilseed rape

The callus pieces from hypocotyl segments of winter oilseed rape were cultured on different concentrations of NAA and BAP (Table 3.8) to study the shoot regeneration. None of the callus pieces showed shoot formation on any of the growth regulator concentration studied. Few of the callus pieces were showing nodular structure but none of them developed into shoots after even allowing longer time.  $\chi^2$ -test showed no significant differences for shoot regeneration at different growth regulator concentrations.

#### 3.4.4. Shoot regeneration in *Brassica rapa* (R.C.)

As already mentioned in the section of 3.2.3., none of the explant pieces, on any of the growth regulator combinations, were able to regenerate shoots. Similar results were obtained when primary callus (callus derived from the hypocotyls explants) was investigated for shoot differentiation. None of the callus pieces, on any of the growth regulator concentrations showed any sign of differentiation.

In case of winter oilseed rape and *Brassica rapa*, where shoot regeneration was not observed using NAA and BAP, other growth regulators were also studied for regeneration. NAA and/or IAA (0.1, 0.2, 0.5, 1, and 2 mg l<sup>-1</sup>), in combination with kinetin or zeatin (1, 2, 3, 4, and 5, mg l<sup>-1</sup>) were studied in different combinations for shoot regeneration from these two species. No shoot formation was observed from any of the species on any of growth regulator combination studied. On some combinations with low NAA and high zeatin embryo-like structures were observed from both of the species. Attempts were made to germinate these embryo-like structures on solid as well as liquid MS-medium, without any growth regulators. None of these embryo like structures gave rise to shoots, but many developed roots. Rooted embryos were put on different media types with and without growth regulators but there was no subsequent regeneration of shoots.

### 3.5. Root formation on the regenerated shoots

After obtaining successful shoot formation the next requirement of any *in vitro* culture programme is rooting of the regenerated shoots. Many researchers have already reported estimation of growth regulators for root formation. In almost all the reports it has been demonstrated that high auxin concentration with no or very low cytokinin concentration, enhances rooting (Flick *et al.*, 1983; Singh *et al.*, 1991). Many media with different growth regulators have been found to be successful for root formation from the regenerated shoots in *Brassica* species. Some of the media with growth regulators found to be successful for root regeneration were MS-medium with IBA+NAA (Singh, 1988), MS-medium with IBA (Glendening and Sjolund, 1988), MS-medium with NAA (Murata and Orton, 1987) B5-medium with IBA (Yang *et al.*, 1994), MS-medium without growth regulators (Hansen and Earle, 1994), prolonged culture on shoot regeneration media (Jain *et al.*, 1988) and 1/2 strength MS-media without any growth regulators (Yang *et al.*, 1991).

To study shoot formation from regenerated shoot on different media a large number of regenerated shoots should be in stock for a formal experiment. In this project it was not possible to arrange for a large number of regenerated shoots of the same age at the same time. Therefore root formation from the regenerated shoots were investigated, informally, at different times depending on the availability of the shoots.

#### 3.5.1. Procedure

Regenerated healthy shoots of approximately same age, size and morphology were harvested. Abnormal leaves attached to the shoots were removed. The culture media studied were, prolonged incubation on the regeneration media, Full strength MS-media without any growth regulators, 1/2 strength MS-media without any growth regulators, MS-media supplemented with 1 mg l<sup>-1</sup> of NAA and MS-media supplemented with 1 mg l<sup>-1</sup> of IAA. About 50 ml of the media was dispensed in to glass jars. Shoot were cultured in the culture media vertically with about 5 mm basal part in side the media. Shoot were incubated in the culture room and were observed weekly.

#### 3.5.2. Results

Roots were produced at the base of the regenerated shoots from the prolonged culture on the regeneration medium, without subculturing for two months. But when the regenerated shoots were subcultured again on the regeneration medium, the

number of shoots were increased but no root were obtained. Root formation in the former case might be the result of exhaustion of nutrients from the medium. It took a long time for root formation on the regeneration medium.

Successful root formation was observed on the culture medium with  $1 \text{ mg l}^{-1}$  of IAA only but the shoots started to elongate. New leaves were narrow and thin in comparison with similar leaves on normal regenerated plants. After some time leaves at the margins and shoot meristems started to wilt and die. On the culture media with only NAA, callus growth was observed at the base of the regenerated shoots. Roots started to emerge from the callus. Shoots and leaves started to die and at the end there was only callus with roots.

Root formation on the full strength as well as on  $1/2$  strength MS-media, with no growth regulators, was successful. Full strength MS-media delayed root formation and took twice the time taken by shoot grown on the  $1/2$  strength media, to produce roots. From these observations it was decided that  $1/2$  strength MS-media without any growth regulators would be used as rooting media for this project.

### 3.6. Discussion

The aims of the different experiments described in this chapter were to select an explant source suitable for callus induction, subsequent callus culture and plant regeneration, and to optimise levels of auxin and cytokinin for callus induction, subsequent callus cultures, shoot regeneration and root formation on the regenerated shoots. Results achieved from the different experiment will be discussed under this section.

#### *Choice of the explants*

The first observation from these studies was that all the explants, studied, were able to induce callus. Statistical analysis for callus induction ability showed no significant differences among the explant types. Millam *et al.*, 1991 also obtained the similar results from their study on rapid cycling accessions of *Brassica oleracea*. They compared different explant sources i.e. internodal sections, leaf discs, petioles and floral sections and observed successful callus induction from all types of explants under study, although with varying degree. Previous work involving tissue culture in *Brassica* species also support these findings. Almost all plant organs of different *Brassica* species have been successfully used by many researchers, depending on their choice or the need of the project (Table 3.10).

From the above discussion it can be concluded that callus induction can be achieved from any plant organ of brassicas and any explant source can be used in tissue culture programme depending on the special needs of the project and availability of the explant. Therefore the decision as to which explant to use was left on the response of explants to shoot regeneration.

While studying the shoot regeneration from different explant type it was observed that growth regulators, explants type and the species or genotypes, all effect the efficiency of shoot regeneration. Neither all the species nor all the explants were able to show regeneration. None of the explants of *Brassica rapa* showed regeneration on any of the growth regulators combination. Winter oilseed rape showed very poor regeneration, whilst curly kale showed the best regenerative ability among the species studied. Genotypic influence on *in vitro* morphogenesis has already been realised in this genus (Fazekas *et al.*, 1986; Palmer, 1992) and it will be discussed in more detail later on in this chapter.

**Table 3.10. Explant sources used successfully in different *in vitro* studies involving *Brassica* species.**

Explant Source	Species/Genotype	Study	Reference
Cotyledon	<i>B. campestris</i> spp. <i>pekenensis</i>	Role of Poly amines on shoot morphogenesis	Chi <i>et al.</i> , 1994
	<i>Brassica</i> spp	Genotypes and media effect	Jain <i>et al.</i> (1988)
	<i>B. oleracea</i> cv. Hydori	Callus, root and shoot formation	Schwammenhöferová-Stranská (1982)
Floral Meristem	<i>B. oleracea</i> (Cauliflower)	Callus, regeneration	Li and Qui (1981)
Hypocotyl	<i>B. campestris</i> (Yellow sarson)	<i>In vitro</i> growth and regeneration	Singh <i>et al.</i> , 1991
	Interspecific and intergeneric hybrids	Regeneration from callus	Hegazi and Matsubara, 1991
	<i>B. juncea</i> cultivars	Variation for regeneration among cultivars	Pental <i>et al.</i> , 1993
	6 <i>Brassica</i> species	Effect of genotype	Dietert <i>et al.</i> (1982)
Leaf	<i>B. oleracea</i> (var. botrytis)	Somatic embryo genesis	Pareek and Chandra (1978)
	<i>B. juncea</i> (Indian mustard)	Role of ethylene on cell differentiation	Pua and Chi, 1993
Leaf Vein	<i>B. oleracea</i> (var. botrytis)	Genetics of growth and differentiation	Buiatti <i>et al.</i> , 1974
Meristem tips	White cabbage	Rapid propagation	Walkey <i>et al.</i> (1980)
Petiole	<i>B. oleracea</i> var <i>italica</i>	regeneration	(Millam <i>et al.</i> , 1991)
Stem Parts	<i>B. oleracea</i> (Cauliflower)	Metal toxicity	Barker, 1972



Regenerative ability observed in this investigation was different from different explant sources. Leaf discs did not show any regeneration, from any of the species studied. In general hypocotyl explants were the most successful explant type for regenerative ability, whilst petioles showed a poor regenerative ability. Statistical analysis showed significant differences among explants for individual genotype as well as for the explants in general regardless of species. Previous work on *Brassica* species also show differences in the efficiency of different explants for shoot regeneration. Some of the researchers who have described variability among explants for regenerative ability are Biber and Reynolds (1983), Lazzeri and Dunwell (1986), Msikita and Skirvin (1989), Millam *et al.* (1991) and Khehra and Mathias (1992). Many of these also found the hypocotyls to be the most successful explant type for regeneration. Lazzeri and Dunwell (1986) tested root segments; hypocotyl segments, leaf disc and cotyledons for regeneration on different types and levels of growth regulators and reported that hypocotyls were the most regenerative explants and frequently more than 80% of the total explant segments produced shoots. Similar results were also observed by Msikita and Skirvin (1989) in *Brassica oleracea* var. *trunchuda*. Not only in brassicas, in other crop species as well, the hypocotyl was found the most successful explant for shoot regeneration ability, including *Capsicum* (Fari and Gako, 1981) and *Glycine* (Kameya and Wildholm, 1981).

Although it is clear that in this study, as with others, hypocotyls were found to be the most successful of the explant type studied, before making a final decision on the choice other prerequisites for an explant type (e.g. easy handling, quick supply in sufficient quantity, sterilisation etc.) should be taken into account. Hypocotyls are one of the most suitable explant type regarding quick supply in sufficient quantity. Within a week, sterile explants in sufficient quantity can be taken, provided sufficient seed is present in stock. Furthermore handling of the hypocotyls is also easy. Hypocotyls are normally taken from *in vitro* grown seedlings. Seeds are sterilised before sowing therefore, no need to sterilise the hypocotyls again. Furthermore seeds are not as delicate as other plant parts, therefore there is less fear of damage during sterilisation process.

Due to the success of the hypocotyl in tissue culture, ease of handling and quick supply, most researchers, working on brassicas prefer the use of hypocotyls to use as model explant source for different tissue culture studies, especially regarding regeneration. Some of the workers who have already used hypocotyl explants successfully are mentioned in Table 3.11 as example.



Table 3.11. Successful use of hypocotyl, as explant source for different *in vitro* culture studies in genus *Brassica*.

Study	Species /Genotype	Reference
Callus + Differentiation	<i>B. campestris</i> (yellow sarson)	Singh <i>et al.</i> (1991)
Callus, root and shoot formation	different <i>Brassica</i> species	Murata and Orton (1987)
Callus + Suspension Cultures	<i>B. campestris</i> (R.C)	Lentini <i>et al.</i> (1986)
Effect of genotype	6 <i>Brassica</i> species	Dietert <i>et al.</i> (1982)
Effect of sodium sulphate	<i>B. napus</i> cv Wester	Chandler <i>et al.</i> (1986)
Growth regulator for shoot and root formation	<i>B. oleracea</i>	Neera-Pradhan and Rajbhandry (1991)
Regeneration	<i>B. nigra</i> (IC 257)	Gupta <i>et al.</i> (1990)
	Different hybrids of <i>Brassica</i>	Hegazi and Matsubara (1991)
	<i>B. oleracea</i> var. <i>tranchuda</i>	Msikita and Skirvin (1989)
	<i>B. campestris</i>	Palmar (1992)
	<i>B. oleracea</i> Var <i>italica</i>	Lazzeri and Dunwell (1986)
	<i>B. carinata</i>	Yang <i>et al.</i> , (1991)
Regeneration from protoplasts	<i>Brassiciceae</i>	Glimelius (1984)
	<i>B. oleracea</i> spp. <i>italica</i>	Kao <i>et al.</i> (1990)
Selection for oxalic acid resistance	<i>B. napus</i>	Chunren and Houli, 1991
Variation among cultivars	<i>B. juncea</i> cultivars	Pental <i>et al.</i> (1993)

### Optimisation of growth regulators for callus induction and subsequent callus cultures.

Hypocotyls were used as the explant source to study callus induction while primary callus, grown from hypocotyl segments, was used to optimise the growth regulators concentration for subsequent cultures. It was observed that when ever auxin and cytokinin were in balance (i.e. NAA/BAP  $\approx 1$ ) there was more callus growth in general as compared with that obtained on other combinations. Generally good callus growth was observed when NAA and BAP concentration was 1-2 mg l<sup>-1</sup> depending on the species. In the literature studied, although no one has concluded that auxin and cytokinin in balance give the best callus yield, overall a similar trend has been shown in most of the investigations (Sharma *et al.*, 1990).

Flick *et al.* (1983) reported that generally a high concentration of auxin and low concentration of cytokinin in the media, promotes abundant cell proliferation with formation of callus. This report was partially supported by the current findings. For curly kale although the optimum concentration for a good callus production was found on the media having auxin and cytokinin in balance (i.e. NAA/BAP = 1), in general media with higher concentrations of NAA as compared with that of BAP also gave better callus yield (Fig 3.2a). But in the case of other species, when auxin was used in higher concentrations although cell proliferation occurred, roots were also induced on the callus. From the findings of these investigation it is not justifiable to generalise the report of Flick *et al.* (1983) for all types of tissue cultures, because explant sensitivity or the original content of endogenous growth regulators may have a great impact on the requirement of growth regulators.

It is universally demonstrated that different genotypes have different responses in tissue culture, especially regarding the type and levels of growth regulator requirement. This may be due to genotype sensitivity or the original content of endogenous growth regulators. Das (1991) investigated callus induction of two *Brassica campestris* genotypes and reported that these two species gave different callus responses to different combinations of auxin in the culture media. Dietert *et al.* (1982) also reported that callus growth depended upon both the genotypes examined, and the culture media. The same was observed in the present study. Statistical analysis, for the studies discussed under section 3.2, showed significant differences between genotypes. While optimising the growth regulator concentration for callus initiation/maintenance production (Section 3.3), the optimum levels of NAA and BAP were found be the same for curly kale and *Brassica rapa* which differed from that for

winter oilseed rape. Curly kale and *Brassica rapa* (R.C.) gave best callus yield when NAA and BAP both were 1 mg l<sup>-1</sup>, while the best callus growth in case of winter oilseed rape was observed at 2 mg l<sup>-1</sup> of NAA and BAP. These results and previous works on *Brassica* species indicate that genotypes may differ in their requirement of growth regulators for callus induction and subsequent callus cultures. Therefore different workers have used different types and levels of growth regulators for callus induction and callus cultures.

After establishing a protocol for successful callus culture, considerable efforts were made to establish a suspension culture. This would allow a study of the effects of different culture systems on *in vitro* selection. But due to unknown causes, suspension cultures could not be established. Possible reasons for this failure to establish viable callus growth in suspension culture, include general culture media required, the viability of the cells, inoculum density, the osmotic potential of the media, the carbon source. Many of these variables were investigated but still it was not possible to establish a viable working protocol. Therefore it became necessary to concentrate all efforts on selection from solid phase callus growth despite awareness of limitations of such culture systems.

#### **Optimisation of growth regulators for shoot and root formation.**

Different *Brassica* species, studied, expressed different shoot regeneration ability. Studies described under Section 3.2.3, using hypocotyl explants demonstrated highly significant differences for shoot regeneration ability from different species. While studying the shoot regeneration using primary callus (Section 3.4.), only one of the three species tested, showed regeneration from callus. The importance of genotype in determining the regenerative ability in *Brassica* species has been observed in many reports. Dunwell (1981) reported that three genotypes of *Brassica oleracea* differed in their response to particular combination of auxin and cytokinin for regeneration. Jain *et al.* (1988) also found considerably varied regeneration frequency between the different species and even the cultivar of the same species.

Different *Brassica* species consist of different cytoplasmic/nuclear genomes. Many workers has found a specific relationship between genomes for their contribution to regeneration. Chopra *et al.* (1986) found consistently superior performance of *Brassica carinata* with 'C' cytoplasm over 'B' cytoplasm. They also observed the inhibitory effect of 'A' genome for morphogenetic response. They further reported that in 'AC' genome 'C' can neutralise the inhibitory effect of 'A',

while in 'AAC' two doses of 'A' genome were strong enough to suppress the morphogenetic response. Murata and Orton (1987) supported these findings suggesting that genes controlling shoot formation are localised in the 'C' genome. Results of the present study also support these findings. In the present study *Brassica oleracea* (Curly kale) carrying 'CC' genome was the most successful genotype for morphogenesis. Shoot regeneration was observed from differentiated tissue (hypocotyl) as well as from dedifferentiated tissue (primary callus). *Brassica napus* (Winter oilseed rape, 'AACC') showed little shoot morphogenesis, only from hypocotyl explants. No shoot formation was observed from primary callus. Possibly equal doses of each genome neutralise the effect of each other and shoot formation observed was very little. The rapid cycle accession of *Brassica rapa* having 'AA' genome (supposed to have an inhibitory effect on morphogenesis) did not show any morphogenesis. These results support the previous findings that genome 'CC' might have the genes for regeneration, while 'AA' has an inhibitory effect on morphogenesis.

The classical findings of Skoog and Miller (1975) that organogenesis in tissue culture is governed by the balance of auxin and cytokinin in the medium cannot be demonstrated universally. In the present study, generally it was observed that when the concentration of BAP (cytokinin) was high with no or low levels of NAA (auxin) more shoot formation was observed. In the inverse conditions of growth regulators the trend was towards root formation. Overall, the same trend has been shown in most of the investigations on tissue culture. According to Flick *et al.* (1983) generally low auxin and high cytokinin concentrations in the medium resulted in the induction of shoot morphogenesis. Lazzeri and Dunwell (1986) also reported that in *Brassica oleracea* shoot formation occurred when the cytokinin:auxin ratio was greater than 1. Singh (1988) reported that lower concentrations of BAP and Kinetin ( $0.5 \text{ mg l}^{-1}$ ) induced roots while higher concentration ( $1\text{--}5 \text{ mg l}^{-1}$ ) induced shoots. Thus it is clear that generally cytokinins induce shoot while auxin promotes root formation for most of the crop species including *Brassica*.

The results also showed that in general, maximum shoot formation was obtained when the media was supplemented with only BAP, without NAA. MS-medium supplemented with  $1 \text{ mg l}^{-1}$  BAP was found the optimum for shoot regeneration in curly kale. Similar results have also been observed by Sharma *et al.* (1990). According to their findings the addition of BAP induced shoot buds differentiation in cotyledon culture of cultivar RIK-81-1 of *Brassica Juncea*. The

highest frequency of shoot buds differentiation occurred with  $5.0 \mu\text{M}$  ( $\approx 1 \text{ mg l}^{-1}$ ) of BAP alone. They also observed that addition of NAA in conjunction with BAP reduced caulogenic response and promoted callus formation and or rooting, which was also observed in the present study. The results of Yanmaz *et al.* (1986) indicated that in culture of cauliflower, BAP promoted shoot proliferation, and the best results were obtained from  $2 \text{ mg l}^{-1}$  of BAP. Many other workers found BAP alone or with a small amount of NAA best for shoot regeneration, among them are Singh *et al.*, 1985 (Cauliflower,  $5 \text{ mg BA l}^{-1}$ ); Shahzadi *et al.*, 1992 (*Brassica juncea*,  $1 \text{ mg BAP l}^{-1}$ ); Msikita and Skirvin, 1989 ( $2 \text{ mg BAP} + 0.1 \text{ mg NAA l}^{-1}$ ); Nera-Pradhan and Rajbhandry, 1991 ( $1 \text{ ppm. BAP} + 0.01 \text{ ppm NAA}$ ); Pawlowski, 1990, ( $2 \text{ mg BAP l}^{-1}$ ) and Yang *et al.*, 1991 ( $2 \text{ mg BAP} + 0.01 \text{ mg NAA l}^{-1}$ ). Only a few reports were found to contradict these findings. Singh *et al.* (1991) reported that regeneration frequency was highest in medium containing  $2 \text{ mg NAA}$  and  $1 \text{ mg BAP l}^{-1}$ . George and Rao (1983) observed that maximum regeneration with BAP ( $0.2 \text{ mg l}^{-1}$ ) alone was 17% while with the combination of BAP and NAA, 95% callus formed shoots in cotyledon cultures of *Brassica juncea* var. RAI-5. The reasons for these contrasts in results may include differences in plant material or other growing or environmental conditions.

As already discussed auxins promote rooting, but according to the literature available, rooting can be achieved on different types of media with or without growth regulators. In the present study rooting was observed on many growth regulator combinations, and even with no growth regulators. Among them, 1/2 strength MS-media without growth regulators was found the most suitable to use in this project. Yang *et al.*, (1991) also used half-strength MS-medium without any growth regulators, for rooting of the regenerants of *Brassica carinata*. Growth regulator-free, half-strength MS-medium has also been found successful for rooting of the *in vitro* regenerated shoots in other crop species as well such as *Cercis siliquastrum* (Grubisic and Culafic, 1984).

In general rooting was more efficient on the media with auxins only, but the shoots were not healthy and sometimes died after some time. Different workers while working on *Brassica* species, have successfully used different types of rooting media such as prolonged culture on shooting media itself (Jain *et al.*, 1988); rooting in sand after treating the regenerated shoots with IAA (Amatya and Rajbahandry, 1991; Neera-Pradhan and Rajbhandry, 199; Poleman-Stephenson *et al.*, 1991); MS-media + NAA (Malik, 1993); MS+IBA (Msikita and Skirvin, 1989); MS+IAA (Bajaj and

Nietsch, 1975; Baburaj and Thamizhchelvan, 1991);  $\frac{1}{2}$  MS+IAA (Shahzadi *et al.*, 1992);  $\frac{1}{2}$  MS+NAA (Zhong *et al.*, 1993); modified MS-media only (Lazzeri and Dunwell, 1984a; Antonetti and Pinon, 1992).

The conclusions drawn from these investigations are that, genotypes, explant source and media composition, may all effect different stages (e.g. callus production; regeneration) of a tissue culture programme. Among the explant sources, hypocotyls gave the best response in tissue culture, especially regarding regeneration. In general, auxin and cytokinin in balance favour callus growth, while high auxin with low or no cytokinin promote rooting and high cytokinin with low or no auxin enhance shooting.



## 4. *IN VITRO* CALLUS SELECTION FOR SALT TOLERANCE.

### 4.1. Introduction

Extensive studies conducted during the last decade have shown that the cell cultures may undergo various morphological and genetic changes (D'Amato, 1985; Evans, 1986; Jain *et al.*, 1989; Yamashita, *et al.*, 1991; Bajaj, 1990) and there has been increasing awareness that this variation may have value for plant improvement (Bajaj, 1990). The potential for the selection of traits of agronomic value using plant tissue culture is well documented (Duncan and Widholm, 1986; Evans, 1989; Collin and Dix, 1990), and has already been discussed under Section 1.4.

Many abiotic antimetabolites have been used successfully in different *in vitro* selection programmes. Such antimetabolites include amino acids analogues (Wakasa and Widholm, 1987), antibiotics (Flick, 1983; Maliga, 1984), herbicides (Jordan and McHughen, 1987), phytotoxins (Larkin and Scowcrof, 1983), heavy metals (Khadeeva *et al.*, 1985; Turner and Dickinson, 1993) and mineral salts (Tal, 1990). Excess of salts in soils is one of the major problems of agricultural land world-wide, and salinity is a significantly limiting factor to agricultural productivity. Excessive salinity within the root zone reduces plant growth. At low concentration salt suppresses plant growth but at higher concentrations can cause death (Shannon, 1984).

Ions that contribute to soil salinity include  $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{HCO}_3^-$ ,  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and rarely,  $\text{NO}_3^-$  or  $\text{K}^+$  (Berstein, 1975). The salts of these ions occur in highly variable concentrations and proportions. Among these salts are NaCl,  $\text{Na}_2\text{SO}_4$ ,  $\text{CaCl}_2$  and KCl. NaCl is one of the major salts present in saline soils and effects damage to plant growth in many ways. In *Brassica* species different selective agents have been used for selecting salt resistance cell lines *in vitro*. These include as sodium sulphate (Chandler *et al.*, 1986), sodium chloride (Jain *et al.*, 1991), and sea water (He and Cramer, 1993). To date most selections using tissue cultures have employed sodium chloride as the selective agent (Stavarek and Rains, 1984; Jain *et al.*, 1991; Kirti *et al.*, 1991b). This reflects the reality that sodium chloride is the most widespread chemical, in saline soils, inhibiting plant growth (Läuchli and Epstein, 1990). For this

study as well, it has been decided that NaCl would be used as selective agent for *in vitro* selections.

To describe the studies related to *in vitro* selection, this chapter is split in to three sections. The first section, focuses on the establishment of a dose response curve for NaCl. The effect of different levels of NaCl has been studied on different culture systems i.e. seed germination and seedling growth, callus induction from hypocotyl segments and subsequent callus cultures. The second section is related to the *in vitro* selection of callus of different *Brassica* species for NaCl tolerance and the effect of selection on salt tolerance. Finally the third section deals with the studies related to stability and genetic nature of salt tolerance in the selected cells.



#### 4.2. Dose response curve of NaCl for different cultures in *Brassica* species

Determination of the selective concentration, of the selective agent, is an initial requirement for any *in vitro* selection programme. Different researchers have used different approaches to decide on the selective concentration. According to Dix (1986) the selective level generally chosen is one just above that at which complete growth inhibition occurs. In this case the selective concentration should be slightly more than lethal concentration. While Ahmad *et al.* (1991) used a concentration, at which 100% embryo kill was obtained, as the selective concentration to select *Brassica napus* embryos for herbicide resistance. Although these approaches have been used successfully, by many researchers, there is very little chances of cell survival. Due to limited chances of cell survival this approach is expected to take more time to select enough material for further studies.

An other possible approach could be that the selective concentration should be the 'highest concentration' allowing cell growth (Tal, 1990). This suggests that the selective concentration should be slightly less than the lethal concentration, and which results in the death of only the sensitive cells. This approach gives more survival chances to the cells under selection pressure. This approach has already been found to be valuable by a number of worker groups (Merrick and Collin, 1981; Jain *et al.*, 1986; Beloualy and Bouharmont, 1991), and has been adopted as the approach to follow in this study.

The selective condition should be determined via growth tests, of the material to be selected, performed under the exact condition anticipated for selection (Dix, 1986). The selective level is normally investigated by exposing the material, to be selected, to a range of concentrations of the selective agent. A dose response curve is established, and from this a selective level is determined. In this project, callus of different *Brassica* species, under study (Section 2.2), will be subjected to selection against NaCl. Therefore the final decision for the selective level of NaCl was made by the exposure of callus to a range of NaCl concentrations.

Also included in this part of the study is the effect of different levels of NaCl on seed germination and seedling growth, and callus induction using hypocotyl explant of different *Brassica* species. The aims of these investigations are to allow a comparison of the selective levels of NaCl for differentiated material (hypocotyl) with that of

dedifferentiated cell mass (callus), and to study whether the selective levels determined for the callus is the same as that for the intact plants.

The seed germination and seedling growth tests were carried out in salt solutions without nutrient medium. To study the effect of nutrient medium on osmotic potential of different salt solutions, osmotic potential of *nd s* solutions with and without MS-medium was checked with a Wescor 5100C vapour pressure osmometer (Wescor Inc., Logan, USA.).

#### **4.2.1. Determination of selective concentrations of NaCl for *in vitro* callus selection.**

Selective levels of NaCl for different *Brassica* species, were determined by culturing callus on media with a range of salt concentration. From the results a dose response curve for each species under study was established and finally selective levels were chosen for each species.

##### **4.2.1.1. Experimental procedure**

Hypocotyl explants from all the species under study were cultured on MS-medium, with optimum growth regulator concentrations (Section 3.3), for callus initiation. Primary callus, produced by hypocotyls was harvested and divided into small pieces of approximately 100 mg. MS-media with different salt concentrations were prepared (Section 2.3). The concentrations of NaCl studied to choose the selective level ranged from 0 to 1.75% (w/v) NaCl (increasing with increments of 0.25% NaCl). Culture media were dispensed in petri dishes of 50 mm diameter. Three callus pieces were cultured randomly, in each petri dish. The experimental design used was CRD with 5 replications. Cultures were incubated in the culture room for 4 weeks.

Callus growth was measured by calculating relative fresh weight gain in each petri dish (Section 2.7). Relative increase in callus fresh weight for each treatment was analysed statistically. A dose response curve was established and finally selective levels were chosen for each species under study.

One of the disadvantages in the use of callus cultures for *in vitro* selection is that only a few of the cells in a callus piece are in direct contact with the selective agent. Therefore there is a possibility of cross-feeding between cells which can affect the results and interfere to the dose response curve. To clarify this point two possible selective concentrations were chosen (giving minimum growth and next level below).

The calli harvested from the medium with these two concentrations were re-cultured on the fresh media with the same salt concentrations. The highest concentration which allowed the growth was finally selected as selective concentration.

#### 4.2.1.2. Results

##### **Effect of NaCl on callus growth from primary callus of curly kale.**

Analysis of variance of relative increase in callus fresh weight (Appendix 4.1) showed highly significant differences ( $P < 0.01$ ) among salt concentrations. Fig 4.1(a) shows that first significant decrease in relative fresh weight was observed when the NaCl concentration was raised from 0.75% to 1%. At the concentrations above 1% NaCl, although there was further decrease in relative fresh weight gain, this decrease was not significant. Comparisons of mean relative fresh weight gain on different salt concentrations (LSD=1.134,  $P=0.05$ ), showed that callus growth on media with concentrations of 1% NaCl, 1.25% NaCl and 1.5% NaCl showed no significant differences. No callus growth was observed at 1.5% NaCl and 1.75% NaCl, and the callus on these media turned brown and died.

The highest concentration of NaCl which allowed the growth of curly kale callus was 1.25% NaCl. Therefore callus from the medium with 1% NaCl and 1.25% NaCl was harvested separately and sub-cultured on media with the same salt concentrations. The culture medium with 1.25% of NaCl did not allowed any further growth and the callus started to turn brown. The callus grown on 1% NaCl continued to grow, although very slowly. These results indicated that 1% NaCl could be used effectively as selective concentration for callus selection in case of Curly kale.

##### **Effect of NaCl on callus production from primary callus of winter oilseed rape.**

Analysis of variance (Appendix 4.2) of relative fresh weight gain, revealed highly significant differences ( $P < 0.01$ ) among salt concentrations. Fig 4.1(b) shows that the first substantial decrease in callus growth was observed on the concentration of 0.25% NaCl. An other significant decrease in callus growth occurred when NaCl was increased from 0.5% to 0.75%. Above this concentration, no significant decrease in callus growth was occurred. At the concentration of 1.25% NaCl and more, the callus pieces turned brown and died. To confirm the highest concentration which allowed callus growth, calli from the media with 0.75% and 1% NaCl were sub-

cultured at the same salt concentrations again. The callus on the medium with 1% NaCl could not survive. While the cultures on the medium with 0.75% of NaCl continued with slow growth. These results indicated that winter oilseed rape may be more sensitive than curly kale for NaCl and the selective concentration chosen for winter oilseed rape callus was 0.75% (w/v) NaCl.

#### **Effect of NaCl on callus production from primary callus of *Brassica rapa*.**

Analysis of variance of relative fresh weight gain revealed highly significant differences among the treatments (Appendix 4.3). Fig 4.1(c) shows that up to 1% of NaCl there was substantial decrease in relative fresh weight gain and at the concentrations above that, no significant decrease was observed.

Callus on the media with  $\geq 1.25\%$  NaCl turned brown and died. At 1% NaCl callus was still green. When the callus from the media with 1% and 0.75% NaCl was sub-cultured on the media with the same concentrations, the callus at both media continued its growth. NaCl concentration of 1% (w/v) was considered as highest concentration allowing the callus growth. From this study it was concluded that 1% (w/v) NaCl could be used as selective concentration for *in vitro* selection of *Brassica rapa* callus for NaCl tolerance.

In general NaCl concentration  $\geq 1.25\%$  was lethal for callus maintenance from different *Brassica* species studied. Different species differ for salt tolerance in tissue culture. Winter oilseed rape was found to be the most sensitive to NaCl in tissue cultures, while the curly kale was found to be the most NaCl tolerant among the species studied.

The data obtained for the dose response curve was also examined using regression analysis (callus growth against log salt concentrations, Fig 4.1. B). However as already indicated, final selection of dose response curve was made on the basis of visual observation. Fig 4.1(B) shows that relative fresh weight gain in curly kale, at selective NaCl level ( $10 \text{ mg l}^{-1}$ ) was approximately 3.2 as compared with that approximately 4.8 on salt free medium (Fig 4.1-A). It indicates that at selective level of NaCl for curly kale the callus growth was about 70% of the control. In case of winter oilseed rape relative increase was about 0.75 (20%) as compared with 3.7 on control. Relative increase in callus fresh weight shown by *Brassica rapa*, at selective NaCl level ( $1 \text{ mg l}^{-1}$ ) was 2 (33%) as compared with 6 at control medium.

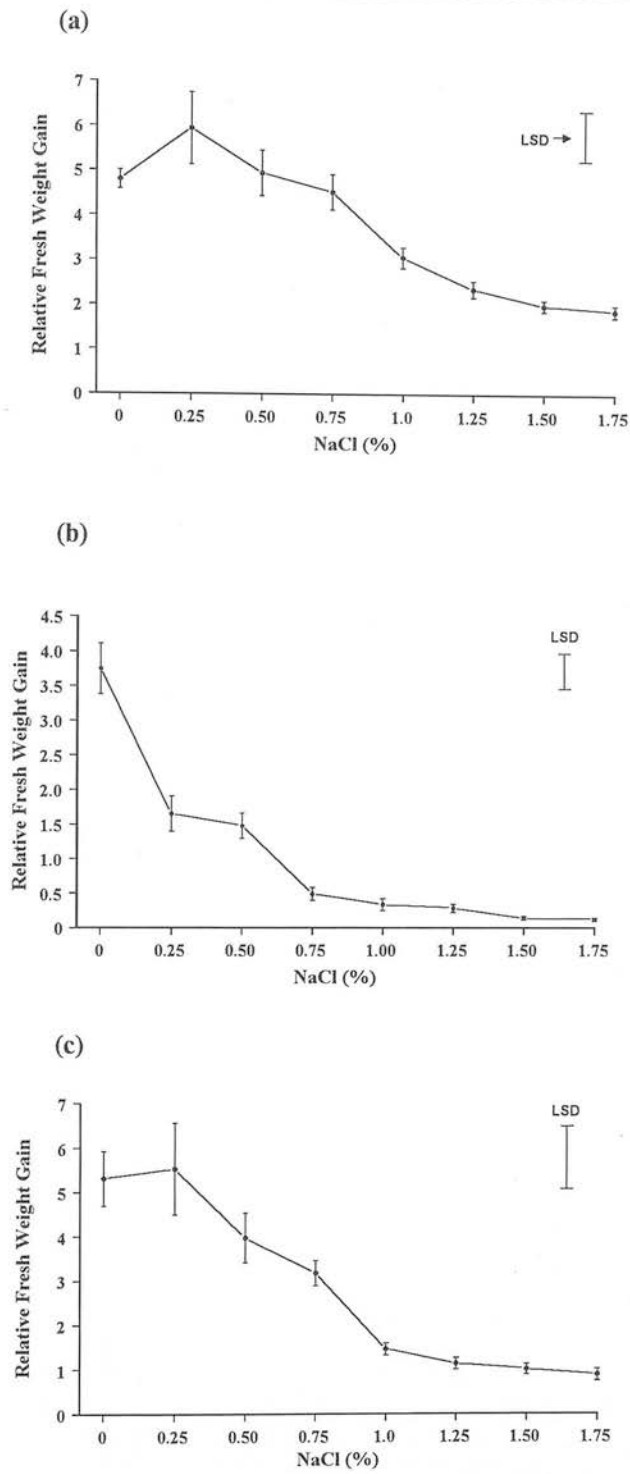
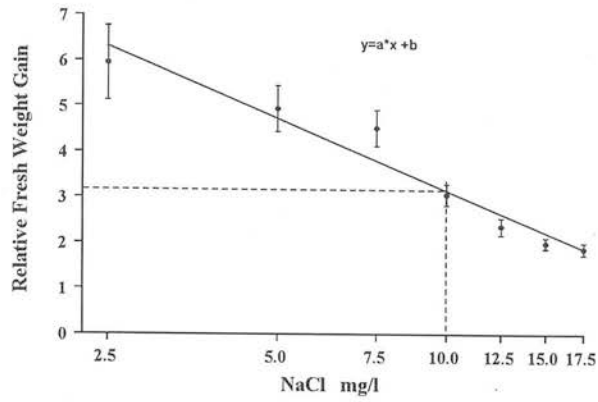
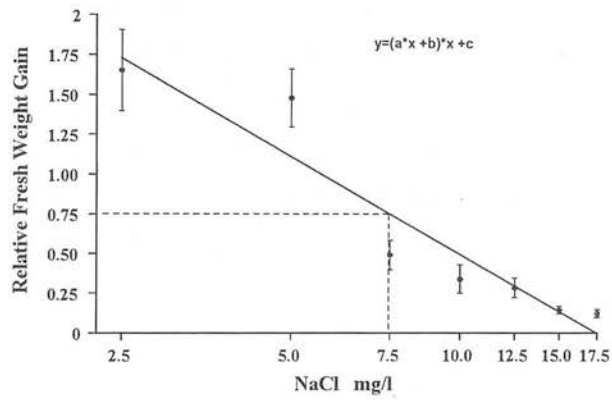


Fig. 4.1-A. Effect of NaCl on callus cultures of different *Brassica* species. Cultures were grown on MS-media supplemented with different NaCl concentrations. (a)Curly kale (b)Winter oilseed rape, and (c)*Brassica rapa*. Mean  $\pm$  SEM,  $n=5$ .

(a)



(b)



(c)

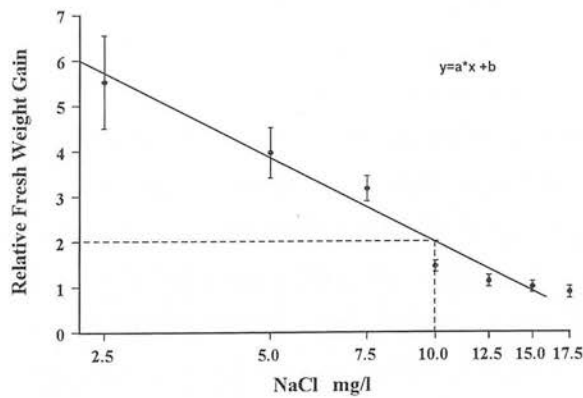


Fig 4.1(B). Dose response curve (regression analysis) of callus growth from different Brassica species for different levels of NaCl. Mean  $\pm$  SEM, n=5.

a:- Curly kale

b:- Winter oil seed rape

c:- Brassica rapa

#### 4.2.2. Effect of different concentration of NaCl on callus induction, using hypocotyl segments of different *Brassica* species, as explant source.

To study the effect of different levels of NaCl on callus induction, hypocotyls were cultured on a range of NaCl levels, and the callus induced in each treatment was observed. The concentration of NaCl studied ranged from 0 to 1.75% (w/v) with dose increments of 0.25% NaCl. The purpose of the current study was to establish a dose response curve for callus induction on different concentrations of NaCl using hypocotyls as an explant source. And to make a comparison between selective levels of NaCl for differentiated and dedifferentiated cell mass.

##### 4.2.2.1. Experimental procedure.

The culture media used was MS-media supplemented with different concentrations of NAA and BAP specific to the species (Section 3.3), 3% (w/v) sucrose, 0.2% (w/v) phytoigel and proposed quantity of NaCl. NaCl was mixed in the culture media, before the adjustment of pH (Section 2.3). Hypocotyl segments from 6 days old, *in vitro* grown seedling, were prepared as mentioned in general materials and method (Section 2.4.3) and cultured on the surface of the media. Cultures were grown in 50 mm diameter petri dishes. Three pieces of explants were cultured in each petri dish, randomly. Experimental design used was CRD, with 5 replications. Cultures were allowed to incubate for 4 weeks, in the culture room. Data was collected by measuring the callus fresh weight in each petri dish (Section 2.7) and analysed statistically using standard analysis of variance.

##### 4.2.2.2. Results

For curly kale, the statistical analysis of callus fresh weight revealed highly significant ( $P < 0.01$ ) differences among the levels of NaCl (Appendix 4.4). Generally callus initiation decreased with increasing NaCl concentration. NaCl concentration from 0-0.5% had no significantly different effects on callus induction ( $LSD = 0.2998$ ,  $P = 0.05$ ). Significant decrease in callus initiation occurred when the NaCl concentration was increased from 0.50% NaCl to 0.75% NaCl (Fig 4.2a). Almost no initiation was observed at 1.5% NaCl and 1.75% NaCl, hypocotyl segments were just swollen and turned brown. Although the fresh weight, at the concentration of 1-1.25% NaCl, was not significantly more than that of the lethal one (i.e. 1.5% NaCl or more), slight callus induction was observed. The highest salt concentration which allowed callus induction from hypocotyl explants of curly kale was 1.25% NaCl.





**Plate 4.1. Callus initiation from hypocotyl segments of *Brassica rapa*, on salt free medium (left) and medium with 1% NaCl**

Analysis of variance of callus fresh weight, in the case of winter oilseed rape, revealed highly significant differences ( $P < 0.01$ ) among different salt concentrations (Appendix 4.5). Fig 4.2(b) shows that there was a substantial decrease in callus fresh weight up to the concentration on 0.75% NaCl, afterwards there was no further significant decrease in callus fresh weight with increasing salt levels (LSD=0.393,  $P=0.05$ ). The highest concentration which allowed callus induction was 0.75% NaCl. At the concentration of 1% NaCl or more, no callus induction occurred, and hypocotyl segments turned brown.

Fig 4.2(c) shows the decrease in callus fresh weight with the increase of salt concentration in the case of *Brassica rapa*. Analysis of variance (Appendix 4.6) of callus fresh weight revealed highly significant differences among the levels of NaCl. There was a substantial decrease in callus fresh weight up to the concentration of 0.75% NaCl. As with winter oilseed rape, no further decrease in callus growth was observed with increasing salt levels (LSD=0.367,  $P=0.05$ ). No callus induction was observed at the media with 1% or more NaCl concentration (Plate 4.1).

In general these results indicate that increasing salt concentration has a negative effect on callus induction from hypocotyl explants of different *Brassica* species. The effect of NaCl on callus induction was different for different species; curly kale was the most salt tolerant among the species studied. Generally NaCl concentration from 1% to 1.5% or more were lethal for hypocotyl cultures of different *Brassica* species.



In general, comparison between the selective (lethal) NaCl concentration for callus induction (using hypocotyl explants) and that for subsequent callus culture, using primary callus as starting material (Section 4.2.1), indicates that the responses were similar. There was some inconsistency with respect to the level of lethal NaCl concentrations for different *Brassica* species. Curly kale appears to allow induction of callus (from hypocotyl explants) at a salt level which prevents subsequent callus growth. However in case of winter oilseed rape callus induction (from hypocotyl explants) was inhibited by a level of NaCl that allowed growth of established callus material. In the case of *Brassica rapa* sensitivity was the same for callus induction as for subsequent subculture of callus.

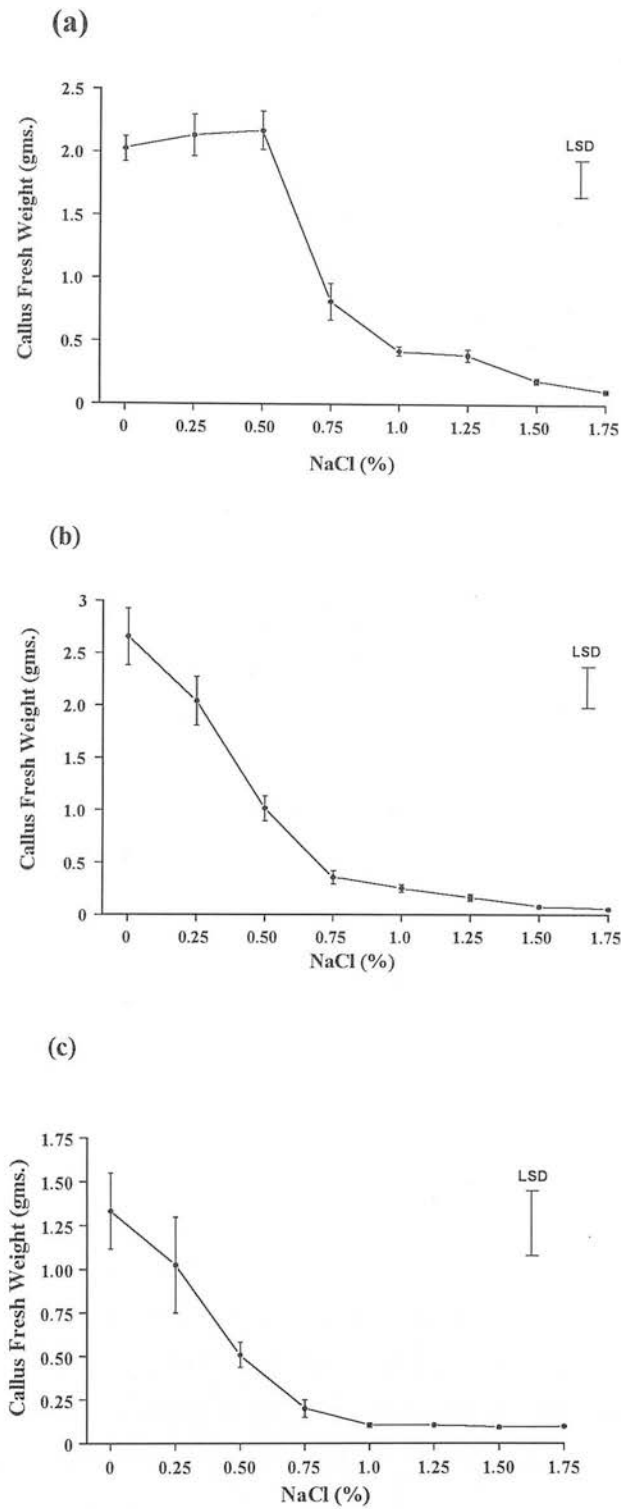


Fig. 4.2. Effect of NaCl on callus induction from hypocotyl explants of different *Brassica* species. Cultures were grown on MS-media supplemented with different NaCl concentrations. (a)Curly kale, (b)Winter oilseed rape and (c)*Brassica rapa*. Mean  $\pm$ SEM, n=5.

#### 4.2.3. Effect of NaCl on seed germination and seedling growth of different *Brassica* species.

To study the effect of NaCl on different *Brassica* species, seeds of these species were allowed to germinate and grow under varying NaCl concentrations. The effect of salt on seedling growth was noted and analysed to differentiate between the species for their inherent level to salt tolerance. The aims of this investigation were to study the effect of NaCl (as a single salt) on the seedling growth of the different *Brassica* species under investigation and to differentiate between the species, for NaCl tolerance. The study was also aimed to make a comparison between selective levels of NaCl for callus cultures, callus induction and on intact plant basis.

##### 4.2.3.1. Experimental procedure

To study the effect of NaCl on different *Brassica* species, the seeds were germinated on a range of salt concentrations. The salt concentrations studied ranged from 0 to 1.75% NaCl, in dose increments of 0.25% NaCl. Salt solutions of different concentration were made by dissolving NaCl in de-ionised water. Un-soaked seeds of different *Brassica* species, under study were, placed on double layer of filter paper (Whatman, grade 18) in plastic petri dishes with 90 mm diameter. Filter papers were wetted with 10 ml of treatment solution in each petri dish. Five seeds were cultured in each petri dish. The petri dishes were sealed with parafilm. Each petri dish was considered as single treatment. The experimental design used was CRD, 2-factor-factorial, with 5 replications. Growth was measured on the basis of seedling fresh weight. Data was collected as seedling fresh weight per petri dish, 6 days after the cultures, and analysed using standard analysis of variance techniques.

##### 4.2.3.2. Results

Effect of NaCl levels, genotypes and their interaction, on the seedling fresh weights, were all highly significantly different (Appendix 4.7,  $P < 0.01$ ). In general, the effect of NaCl was different on seedling growth from the different *Brassica* species. Increasing salt concentration resulted in decreased seedling growth and at certain level of NaCl no seed germination or seedling growth was observed (Fig 4.3, Plate 4.2). The NaCl concentration 1.75% did not allow seed germination in any of the species studied.

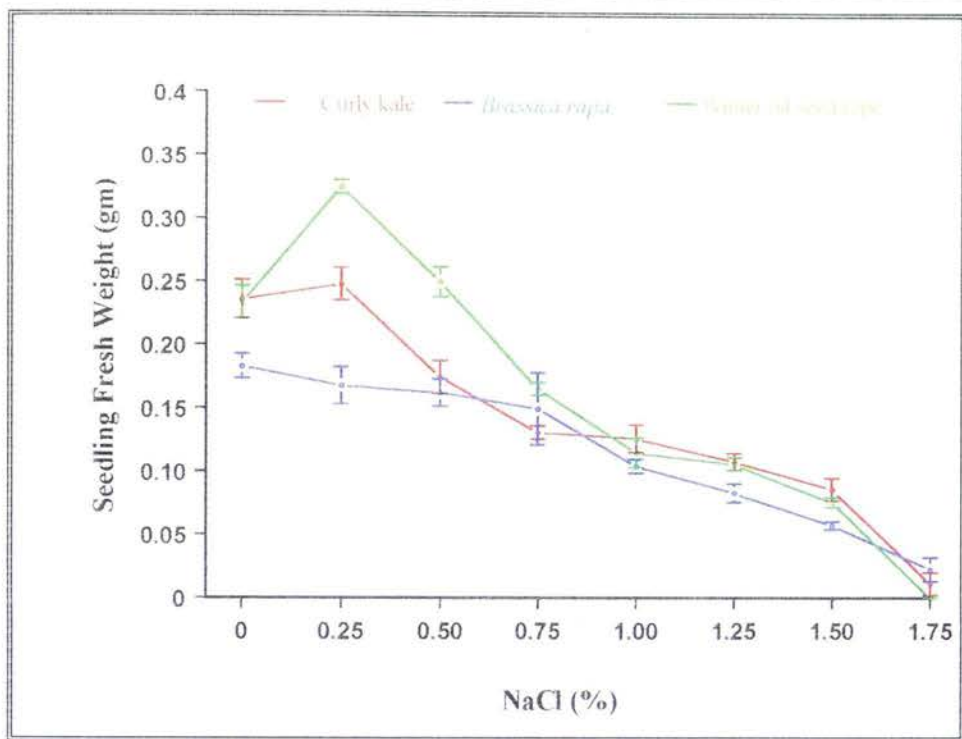


Fig. 4.3. Effect of various levels of NaCl on seedling fresh weight from different species of Brassica. Fresh weight was measured 6 days after seed sowing. Mean  $\pm$  SEM,  $n=5$ .



Plate 4.2. Decrease in seedling growth, in Curly kale, with increasing concentration of salt. NaCl concentration increasing from 0 to 1.75% (w/v) with an increment of 0.25% NaCl (from left to right)

The primary objective of the current study was to investigate the effects of different salt concentration on seedling growth of different *Brassica* species. Since interaction between salt concentration and the species was significant, for more information, the same data was re-analysed for each species separately.

#### **Effect of NaCl on seed germination and seedling growth of curly kale**

Analysis of variance of seedling fresh weight, revealed highly significant ( $P<0.01$ ) differences among the levels of NaCl (Appendix 4.8). No significant change in seedling growth of curly kale was observed with the slight increase (0 to 0.25% NaCl) in salt concentration, but further increase in salt concentration resulted significant decrease in seedling growth. At the concentration of 1.75% NaCl no seed germination was observed.

Seedling growth at 1.25 and 1.5% NaCl was minimum and the difference between mean seedling fresh weight was not significant at these two levels (LSD=0.031,  $P=0.05$ ). Seedling fresh weight at the concentration of 1% NaCl was significantly different from that at 1.5%, but not that at 1.25% NaCl. These results indicate that for curly kale, 1.5% NaCl was the maximum concentration at which seed germination and seedling growth was observed.

#### **Effect of NaCl on seed germination and seedling growth of Winter oilseed rape.**

Analysis of variance showed highly significant ( $P<0.01$ ) differences among the effects of salt concentrations on seedling fresh weight of winter oilseed rape (Appendix 4.9). Seedling fresh weight increased with the slight increase (0 to 0.25%) in NaCl level, but decreased significantly with further increase in salt concentrations. At 1.75% NaCl no seedling growth was observed, and seeds were not germinated properly.

Seedling fresh weights at the concentration of 1.25% NaCl were significantly different from that at 1.5% NaCl, but not significantly different from that at 1% NaCl (LSD= 0.023,  $P=0.05$ ). These results indicate that maximum salt concentration, allowing seedling growth in winter oilseed rape was 1.50% NaCl.

#### **Effect of NaCl on seed germination and seedling growth of *Brassica rapa*.**

Analysis of variance of seedling fresh weight showed highly significant ( $P<0.01$ ) differences among the different NaCl levels (Appendix 4.10). No significant decrease

in mean fresh weight was observed until 1% NaCl (LSD= 0.038,  $P=0.05$ ). Significant differences were also not observed for average fresh weight at the concentrations of 1 and 1.25% NaCl. The results obtained indicated that minimum growth occurred at the concentrations of 1 and 1.25% NaCl in case of *Brassica rapa*. No seedling growth was observed at the concentration of 1.5% and more NaCl.

In general, these results indicate that *Brassica* species differ with respect to salt tolerance. Generally a small amount of salt (i.e. up to 0.25% NaCl) does not effect on plant growth negatively. In some species growth may increase with slight increase in salt concentration. In most of the cases NaCl concentration 1.25% to 1.5% was found to be the maximum concentration allowing seed germination and seedling growth.

While comparing the results obtained from previous studies (Section 4.2.1, 4.2.2, 4.2.3.) it is clear that lethal concentration of NaCl in the case of intact plants was slightly more than that *in vitro* cultures (i.e. for callus initiation and callus cultures). In general, in all the species, these results indicated that intact plants were more salt tolerant than *in vitro* cultures. Lethal concentration for the seedling growth ( $\geq 1.5\%$  NaCl) were slightly more than that for callus initiation and subsequent callus cultures (1-1.5% NaCl). The higher salt tolerance shown by the intact plants as compared with the tissue cultured material might be the result of cellular organisation in intact plants which was absent in tissue cultured material. Another possible reason for this higher tolerance might be the difference in osmotic potential in two NaCl solutions. The osmotic potential of NaCl solution with MS-medium (in case of tissue cultures) was more than that without MS-media (seed germination, Fig 4.4). The difference in osmotic potential was almost equal to 0.5% NaCl in magnitude, a level almost equivalent to the difference in 'salt tolerance' observed, at same level in two different solution.

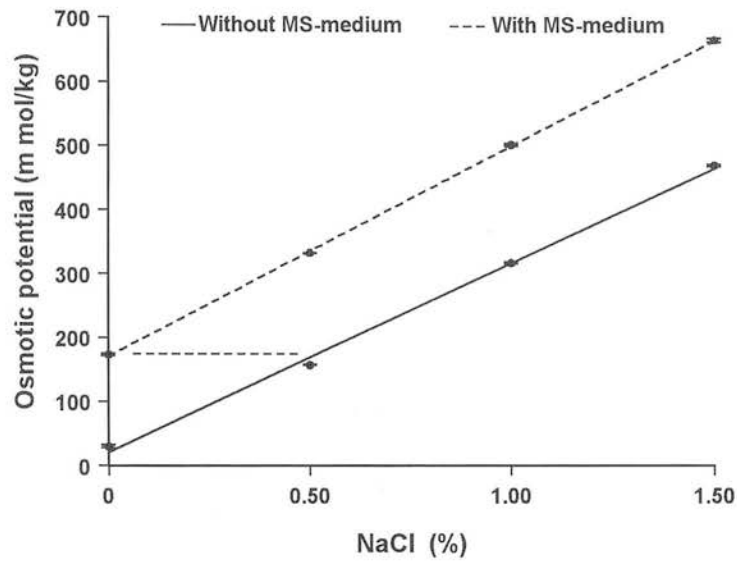


Fig 4.4. Difference in osmotic potential of two types of NaCl solutions i.e. with MS-medium (for *in vitro* cultures) and without MS-medium (for seed germination) used in experiments.



### 4.3. In vitro selection for salt tolerance

There is an increasing awareness of the potential and limitations of the technique of tissue culture for the production of new genotypes with valuable characteristics for agricultural use, particularly in relation to salt resistance (Nabors *et al.*, 1980; Smith and McComb, 1981; Handa *et al.*, 1982b; Nieman *et al.*, 1983; Chandler and Vasil, 1984; McHughen and Swartz, 1984; Lebrun *et al.*, 1985; Pua and Thorpe, 1986; Bajaj and Gupta, 1987; McCoy, 1987a,b). The cell culture approach has been proved effective in obtaining salt resistant cell lines in a considerable number of plant species (Tal, 1990) including *Brassica* species. Attempts to select salt-resistant cells in culture are based on the assumption that cultured cells represent physiologically, at least in part, the cells of the whole plant, i.e., that cellular mechanisms which are responsible for salt resistance in cultured cells may also operate in subsequently derived whole plants (Tal, 1990). Correlation in the response to salinity between whole plant performance and cellular mechanism has been confirmed for several plant species (He and Cramer, 1993).

The purpose of this investigation was to select the cells showing resistance to NaCl and to estimate the tolerance index of callus to selective concentrations of NaCl. Selection was started mainly in the fourth culture cycle (C4). The reasons for delaying the selection up to 4th culture cycle was to give reasonable time to multiply the callus to the required quantity and to generate somaclonal variation.

One of the disadvantages in the use of callus cultures, for *in vitro* selection is that only a small portion of the total cells in a callus piece are in direct contact with the stress condition (Dix, 1986). One way of reducing these defects are to use small callus pieces for selection. Therefore for selection purposes callus would be cut into small pieces of about 60 mg, instead of 100 mg as used in other subcultures.

#### 4.3.1. Experimental procedure

Control and the selective culture media, specific to species, was prepared (Section 2.3) and dispensed into 50 mm diameter petri dishes. Callus of all the species under study was harvested after completing the 3rd culture cycle. Healthy green, fast growing callus was cut in to small pieces ( $\approx 60$  mg) and bulked together. Five pieces of the callus were cultured randomly, in each petri dish. A total of 10 replications were used for each treatment. All the petri dishes were sealed with a double layer of parafilm and randomised in the culture room. After one month of incubation callus growth was measured in terms of relative fresh weight gain. The



callus growth on selective media was compared with that on non-selective medium by applying t-test statistics. Because the selective conditions and the culture requirements were different for different species, results from each species were analysed separately.

Tissue growing on the selective medium was harvested. Any necrotic regions were discarded and only healthy, fast growing tissue was subcultured on to the selective media again, and allowed to grow for a further month. Thereafter tissue pieces still showing growth were selected and maintained on normal non-selective medium. A portion of the selected callus was also grown on selective medium from which a few callus pieces were maintained on salt free medium after each selection cycle. Consequently, different types of callus of the same age from the same source was available, which differed only in their selection period. These different callus type from the same source were used for further study.

#### 4.3.2. Selection of curly kale callus for 1% NaCl resistance

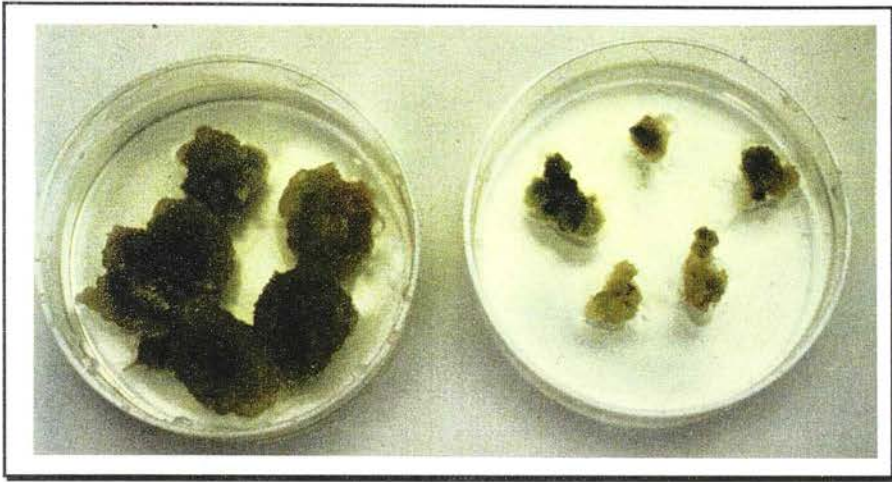
The aims of this study were to select the callus of curly kale, capable of growing on 1% NaCl, and to study the effect of 1% NaCl on callus growth of curly kale callus in 4th culture cycle.

Statistical analysis of relative fresh weight gain revealed highly significant differences in callus response to media type (Table 4.1). Ccallus growth on non-selective conditions was almost double to that on selective conditions (Fig 4.4a, Plate 4.3). The curly kale callus in 4th culture cycle showed 50.82% tolerance index (Section 2.7) for 1% NaCl.

**Table 4.1. Results of different t-tests for callus growths of different *Brassica* species on selective and non-selective culture medium.**

Species	D.F.	t-value
Curly kale	9	3.194 **
Winter oilseed rape	9	4.120 **
<i>Brassica rapa</i>	9	5.115 **

\*\* Highly Significant ( $P < 0.01$ )



**Plate 4.3.** Callus growth from curly kale callus, on salt free medium (left) and medium with 1% NaCl (right).

#### **4.3.3. Selection of winter oilseed rape callus, tolerant to 0.75% NaCl.**

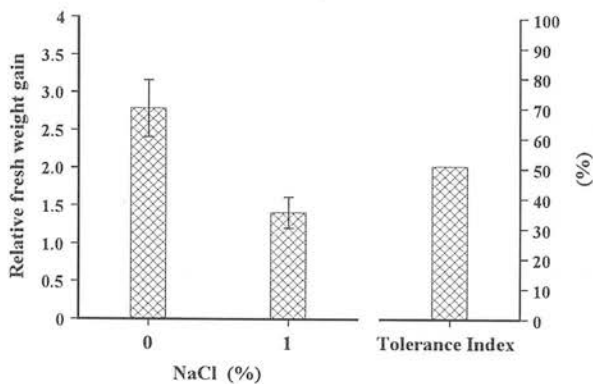
Winter oilseed rape callus tissue were subjected to the selective concentration i.e. 0.75% NaCl. The t-test (Table 4.1) revealed highly significant differences between callus growth on selective and non-selective culture media. The relative increase in callus fresh weight on non-selective medium was about 3 fold that on selective medium. Winter oilseed rape callus, in 4th culture cycle showed 34.64% tolerance index for 0.75% NaCl (Fig 4.4b).

#### **4.3.4. Selection of *Brassica rapa* callus tolerant to 1% NaCl.**

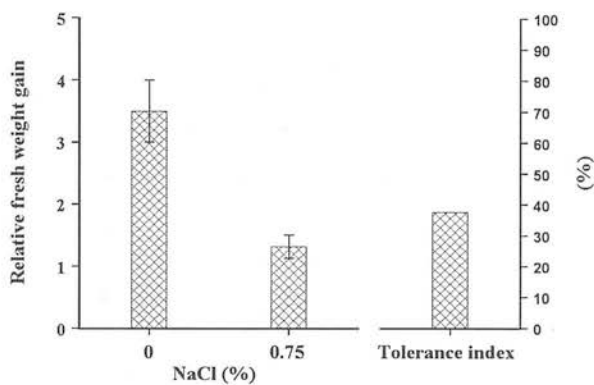
The selective concentration of NaCl chosen for the selection of *Brassica rapa* callus was 1% (Section 4.3.4). The t-test (Table 4.1) revealed highly significant differences between relative increase in callus fresh weight on selective and non-selective culture media. Callus of *Brassica rapa* in 4th cultural cycle showed 44.19% tolerance index.

In general, these results indicated that callus growth from different species, on their specific selective concentrations, ranged from 30-50% of the total callus produced on the control medium. Maximum tolerance index was shown by a curly kale callus at 1% NaCl (50.82%) and minimum by winter oilseed rape at 0.75% NaCl (34.64%).

(a)



(b)



(c)

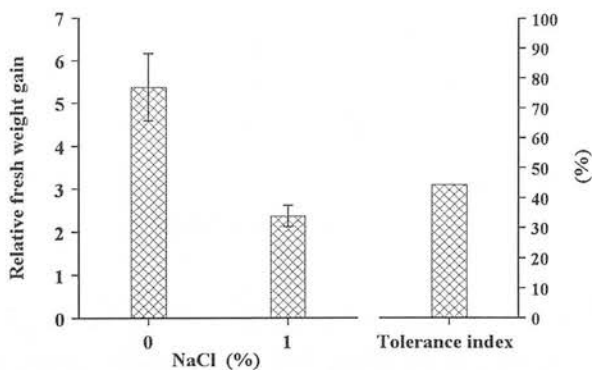


Fig. 4.5. *In vitro* selection of callus of different *Brassica* species. Selection was carried out in 4th culture cycle, at selective concentration of NaCl specific to the species. (a) Curly kale, (b) Winter oilseed rape and (c) *Brassica rapa*, Mean  $\pm$  SEM, n=10.

#### 4.4. Stability for salt tolerance

If callus or regenerated plants derived from the callus, selected for NaCl tolerance, showed more salt tolerance as compared with un-selected callus (or their derived plants) from the same lot, even after culturing on salt free medium for few generations, the selected callus is considered stable for salt tolerance. The same criteria for salt tolerance stability was also used by Jain *et al.* (1989). They confirmed the stability of altered response of the selected clones by growing it on normal medium for three months and then bringing back to the stress medium.

Stability of salt-tolerance in cultures provides an indication of the effectiveness of selection. Ben-Hayyim and Kochba (1982) and Dix and Street (1975) suggested stability as an indicator for the selection of true genetic variants, for the character in study. More clearly, stability for the selected character, which can be observed in regenerated plants and their successive generations is a true indicator for the selection of true genetic variants. Therefore, after the selection of callus at the selective concentrations of NaCl, the next stage of this project was to check the stability of the selected callus for salt tolerance.

Since the regeneration of the plants was not achieved in some of the species under study, in most of the cases of stability of salt tolerance was studied only in the callus phase. However, although *in vitro* regeneration was successful in curly kale, regeneration under selective conditions was not normal (Plate 4.4). On the regeneration medium with NaCl, differentiation started which resulted in abnormal regeneration. Only abnormally, enlarged leaves were regenerated and further shoot elongation and field transfer was not successful. Therefore, field testing and the progeny testing for salt tolerances was not possible in curly kale as well. To study the salt tolerance after regeneration, the regenerated leaves, from selected and un-selected callus, were used to study the salt tolerance *in vitro*.

Different callus type of the same age, which differed only in their selection/maintenance cycles (Section 4.3.1) were used to compare their stability for salt tolerance. Stability tests were performed in different culture cycles which depended on the availability of the callus in sufficient quantity. Stability experiments could not be carried out for all the required culture cycles due to non availability of the callus in required quantity. Sometimes, due to contamination of one or more

treatments, experiments could not be completed. Therefore the results of only those experiment which were completed successfully are provided.

The abbreviations C, M, and S used, are for culture cycles, maintenance cycles on non-selective medium after selection and selection cycle, respectively. These abbreviations has been described in detail under Section 2.8.

#### 4.4.1. Experimental procedure

Different types of selected callus (Section 4.3.1), along with the control, were harvested and cut into small pieces ( $\approx 100$  mg). The pieces from each callus type were bulked together and then divided randomly into two portions. One portion was transferred on the selective medium while the other was cultured on non-selective medium. Cultures were grown in plastic petri dishes of 50 mm diameter. Three pieces of callus were cultured in each petri dish. Experimental design used was CRD, 2 factors factorial, with 5 replications. Cultures were incubated in the culture room and the relative fresh weight gain was calculated after 4 weeks of incubation.

Statistical analysis were carried out using original data, while the tolerance index was calculated on the basis of the average of the 5 replications. The portion of the each callus type cultured on non-selective medium was considered the control to calculate the tolerance index for that type of callus (Section 2.8).

The aims of the following experiments were to investigate the stability of the selected callus for NaCl tolerance, to make a comparison between tolerance of selected and un-selected callus, to differentiate between salt tolerance of differently selected callus and to find out the effect of selection and duration of selection on tolerance index.



(a)



(b)



Plate 4.4. Regeneration from curly kale callus. (a) regeneration on salt free medium and (b) regeneration on the medium with 1% NaCl.

#### 4.4.2. Stability tests for curly kale callus

##### Stability test for different types of curly kale callus in 9th and 14th culture cycle.

Two experiments (i.e. in the 9th and the 14th culture cycles) were carried out to study the stability of the selected callus, for salt tolerance in curly kale. In 9th culture cycle, the callus types investigated were (1) un-selected (control, 8C), (2) callus grown on selective medium for 5 consecutive culture cycles (3 standard cultures+5S) and callus selected for 3 culture cycles and then maintained on non-selective medium for 2 generations (3 standard cultures+3S+2M). Selection in both the cases was started in 4th culture cycle. The callus type studied in 14th culture cycle were, un-selected callus (control, 13C), callus selected consecutively for three culture cycles and then maintained on non-selective medium for 3 generations (7 standard cultures+3S+3M) and the callus selected consecutively for 6 culture cycles (7 standard cultures+6S). Selection, in both the cases, was started in 8th culture cycle and callus was selected at 1% NaCl in culture medium.

In both the experiments, the analysis of variance of relative increase in callus fresh weight revealed (Appendix 4.11 and 4.12) significant differences ( $P < 0.05$ ) between media type. No significant differences ( $P \geq 0.05$ ) between callus type and interaction between callus type and the culture media, on callus growth were found. Non-significant effects of callus type and interaction on callus growth indicated that selection did not have significant effect on callus for 1% NaCl tolerance. On the other hand Fig 4.7(a,b), indicates that callus growth of all types of callus on non-selective medium was not much different from each other, while the callus growth on selective culture medium (with 1% NaCl) was different for different callus type. Selected callus (maintained on the selective medium) showed more callus growth as compared with selected+maintained (on salt free medium) callus and un-selected callus. Increased salt tolerance shown by the selected callus as compared with that from non-selected, indicated that selection might have increased salt tolerance in curly kale, but this increase was not significant statistically.

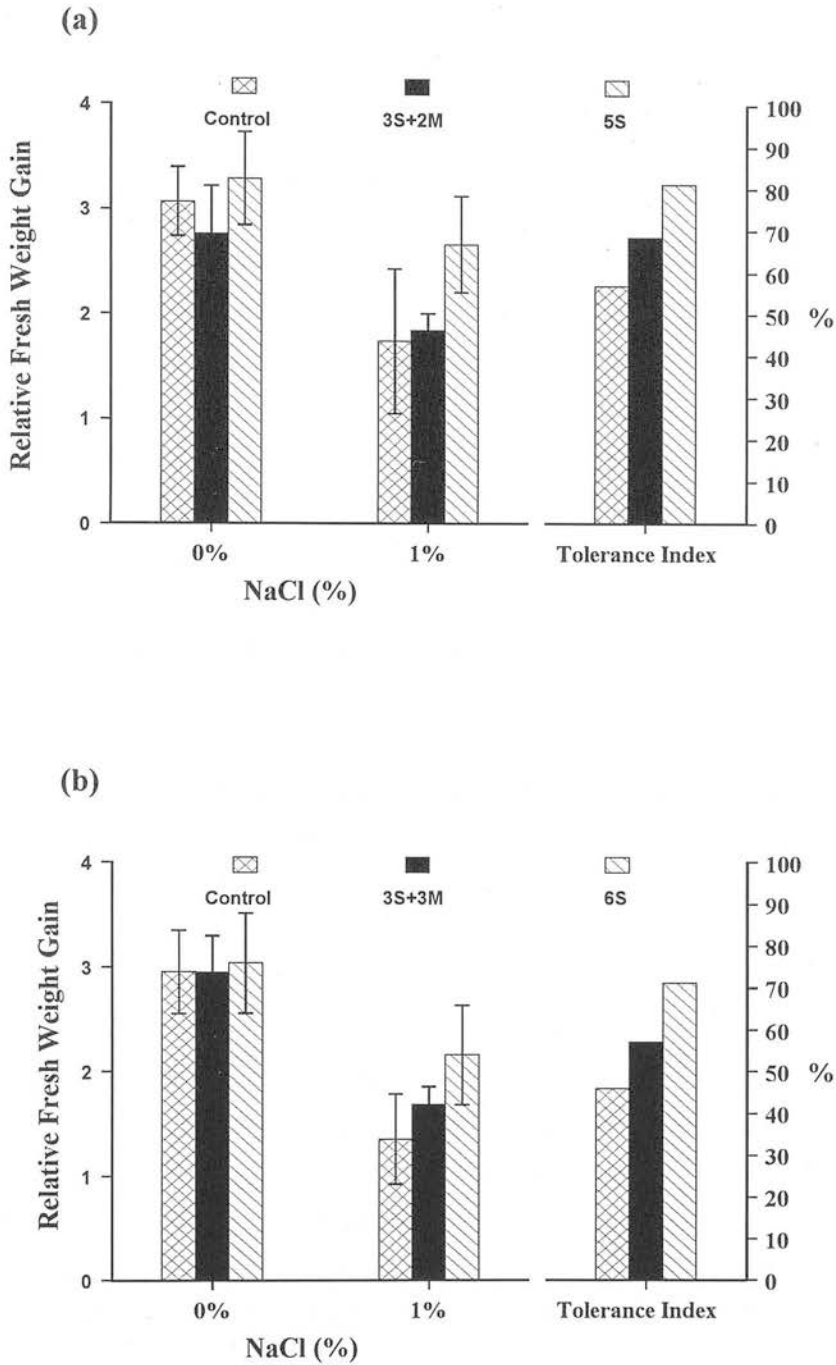


Fig. 4.6. Salt tolerance of different types of selected and un-selected callus of Curly kale. (a) stability test in 9th culture cycle (b) stability test in 14th culture cycle. Mean  $\pm$  SEM,  $n=5$ .



### **Salt tolerance from regenerants of curly kale.**

Regeneration was carried out from selected and un-selected callus of the same age. The regenerants from the selected callus were regenerated under selective conditions, after passing 3 selection cycle on selective medium, in callus phase. Due to the abnormal regeneration on selective medium (Section 4.4) and/or the limitation of appropriate number of regenerated plants, stability tests on the regenerants were carried out only *in vitro*. The test were performed in two steps i.e. by studying callus induction from leaf discs and subsequent callus cultures. In the first experiment leaf discs were punched from the leaves of both type of the regenerants and cultured on selective as well as on non-selective culture medium (Section 2.4.3). In the second experiment callus produced from the leaf discs, on non-selective medium, was then subcultured for further stability tests. After an incubation period of 4 weeks, callus fresh weight from leaf discs cultures and relative fresh weight gain (Section 2.7) from subsequent cultures was calculated and analysed statistically.

The aims of these investigations were to study the stability for salt tolerance after regeneration and to make a comparison between salt tolerance of regenerants from selected and un-selected callus.

In both the experiments analysis of variance (Appendix 4.13 and 4.14) of callus induction and callus growth revealed no significant differences among plant source and interaction between the plant source and the media type. The only significant differences observed were related to the effect of media type. No significant effects of plant source and interaction indicate that selected regenerants were no more salt tolerance, at least *in vitro*, as compared with un-selected one. Fig. 4.6(a) shows that tolerance indices of both type of the plants was almost the same (Plate 4.5). While Fig 4.6(b) shows slightly more salt tolerance from selected regenerants as compared with that from un-selected regenerants. The difference between the tolerance indexes was very small.

Results of stability tests for curly kale indicated that selection did not increase salt tolerance significantly. Although salt tolerance, as shown by the selected callus was more than that from un-selected callus, the difference was not significant.



Plate 4.5. Callus initiation from leaf discs of regenerants of curly kale. Top row: leaf discs from plants regenerants of selected callus. Bottom row: leaf discs from regenerants of non-selected callus. Left: growth non-selective medium. Right: growth on the medium with 1% NaCl.

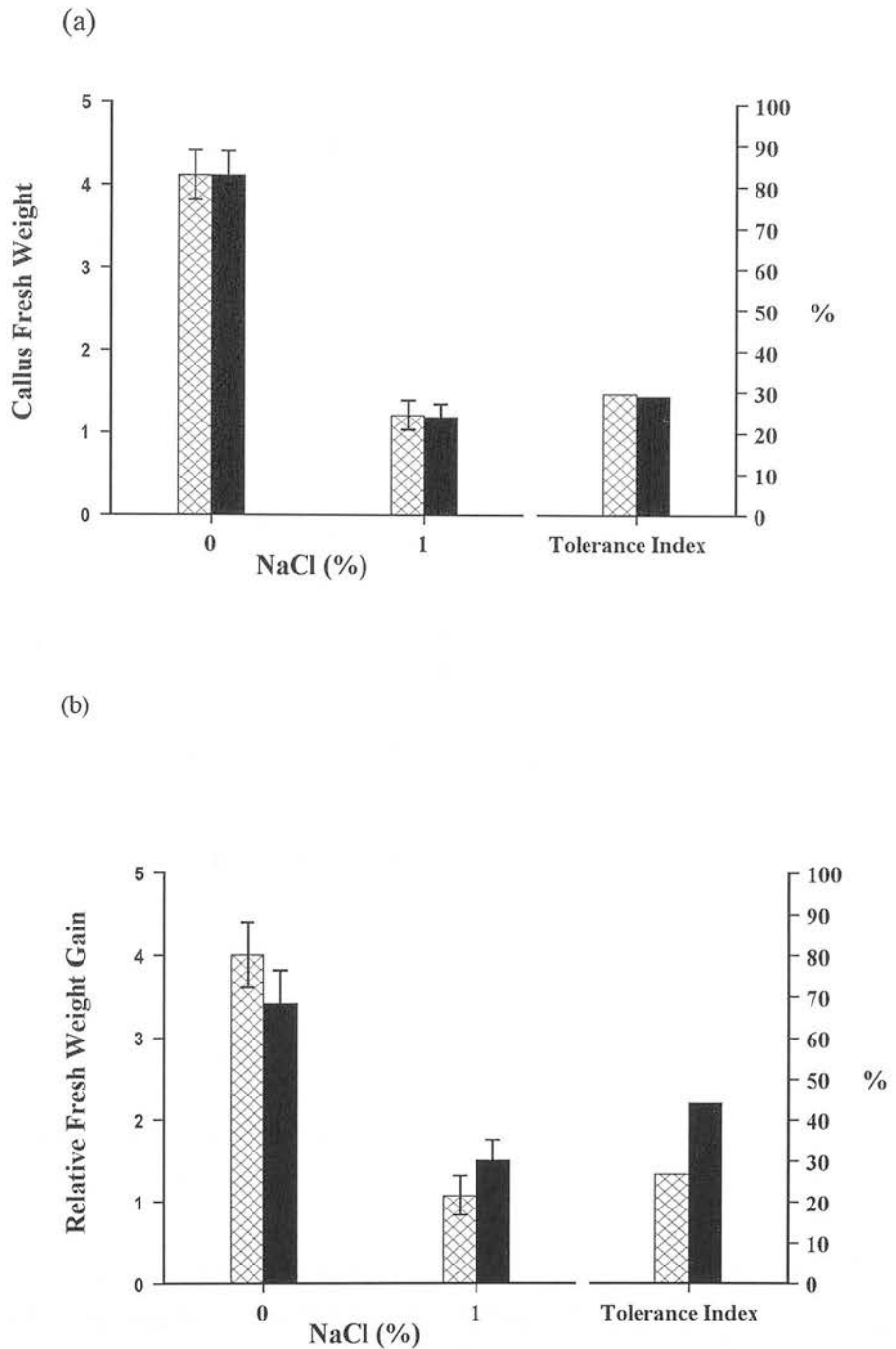


Fig. 4.7. Salt tolerance of regenerants from selected and un-selected callus of Curly kale. ■ regenerants from selected callus, ▨ regenerants from un-selected callus. (a) callus induction from leaf discs, (b) subsequent callus cultures. Mean  $\pm$  SEM,  $n=5$ .

#### 4.4.3. Stability tests for winter oilseed rape

For winter oilseed rape, stability for salt tolerance was studied in two generations only i.e. in 9th and 14th culture cycles. Three different types of calli were studied in the 9th culture cycle, these included (1) control callus (8C), (2) callus selected for three culture cycles and maintained for further 2 generation on non-selective medium (3 standard cultures+3S+2M) and (3) callus selected consecutively for 5 culture cycles (3 standard cultures+5S). Selection in both the cases was started in 4th culture cycle. The callus types studied in 14th culture cycle were, (1) unselected callus (control, 13C), (2) callus selected continually for 3 and generations (10 standard cultures+3S) and (3) callus selected for 3 generation followed by maintenance on non-selective medium for further 3 generations (7 standard cultures+3S+3M). Selection in former case was started in C11 and in later case, it was started in C8. The aims of these experiments were as stated before under Section 4.4.1. As the selective concentration for winter oilseed rape callus was 0.75% NaCl, the same was used to study the stability for salt tolerance.

In both the experiments, the analysis of variance for relative increase in callus fresh weight (Appendix 4.15 and 4.16) revealed highly significant differences ( $P < 0.01$ ) between culture media and non-significant differences ( $P \geq 0.05$ ) between callus type and interaction between callus type and culture media. These results indicated that *in vitro* selection of callus for salt tolerance did not have significant effect on salt tolerance. On the other hand Fig 4.7(a,b) shows that selection had some effect on salt tolerance, although this effect was not significant. Selected callus showed more callus growth on selective medium as compared with that by unselected callus. The tolerance index, calculated for control callus was less as compared with that for selected calli.

These results indicated that selection for 0.75% NaCl did not significantly increased salt tolerance of winter oilseed rape callus. Apparently there was slightly increase in tolerance index due to selection, but this increase was not significant statistically.

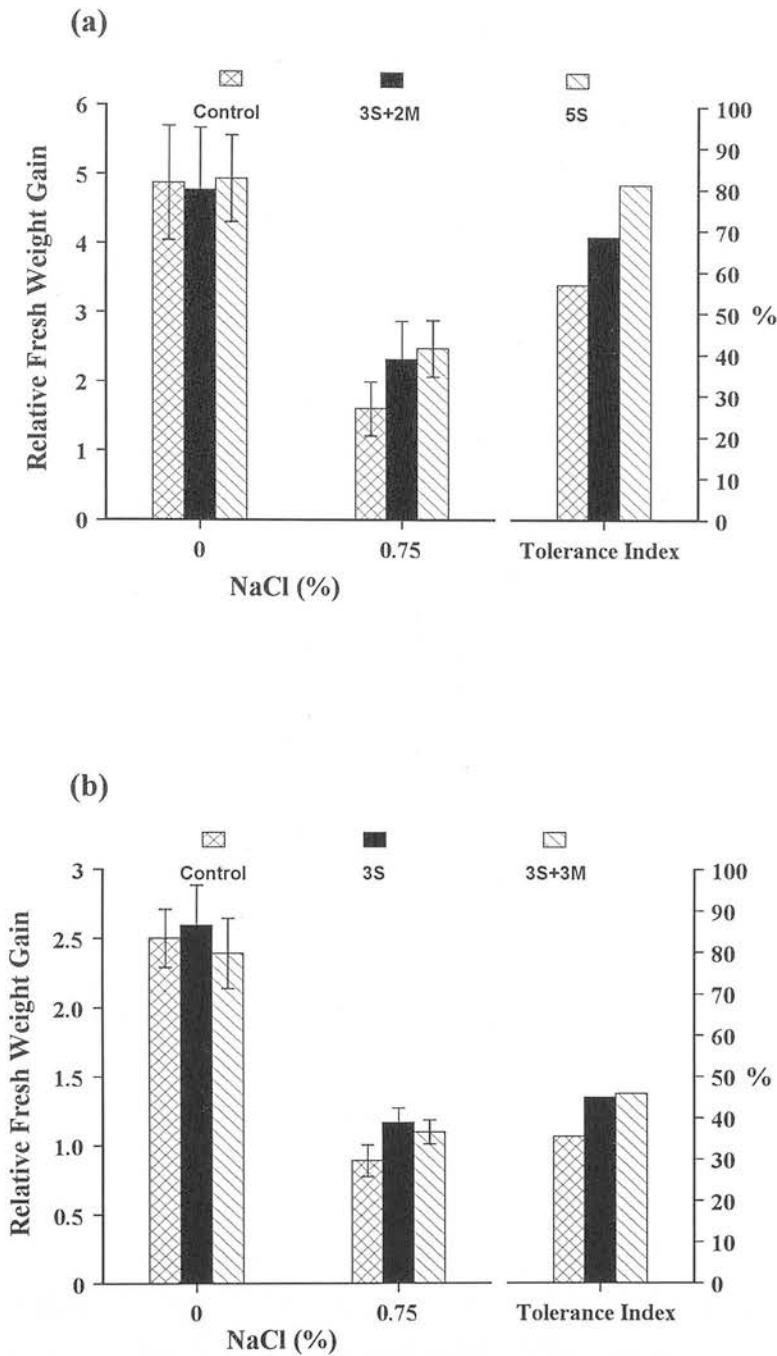


Fig. 4.8. Salt tolerance of different type of selected and un-selected callus of Winter oilseed rape. (a) stability test in 9th culture cycle (b) stability test in 14th culture cycle. Mean  $\pm$  SEM,  $n=5$ .

#### 4.4.4. Stability tests for *Brassica rapa* callus

The cultures of *Brassica rapa* were growing very successfully and fortunately there was less contamination in these cultures, therefore it gave more opportunities to study the stability for salt tolerance in different culture cycles. Stability for salt tolerance in *Brassica rapa* was studied in the 10th, 14th, 16th and 17th culture cycles. In each experiment different types of callus of the same age were studied for salt tolerance. The aims of these investigations were the same as described under section 4.4.1.

Only two callus type were studied in the 10th culture cycle, these included control callus (9C) and callus selected for two generations and then maintained on non-selective medium for further four generations (3 standard cultures+2S+4M). For the selected callus the selection was started in 4th culture cycle. The callus type studied in 14th culture cycle were (1)control callus (13C), (2)callus selected for 3 culture cycles followed by maintenance on un-selected culture medium, for 3 culture cycles (7 standard cultures+3S+3M) and (3)callus selected for 6 generation (7 standard cultures+6S). Two types of callus, studied in 16th culture cycle were control callus (15C) and callus selected consecutively for 7 generations (8 standard cultures+7S). And in 17th culture cycle, callus types studied were (1)control callus maintained on normal culture medium for 16 culture cycles (16C), (2)callus selected for 8 culture cycles and maintained for 2 culture cycles (6 standard cultures+8S+2M), (3)callus selected for 9 generations and maintained for 1 culture cycle (6 standard cultures+9S+1M) and (4)callus selected consecutively for 10 generations(6 standard cultures+10S). Selection for all the selected callus was started in 7th culture cycle.

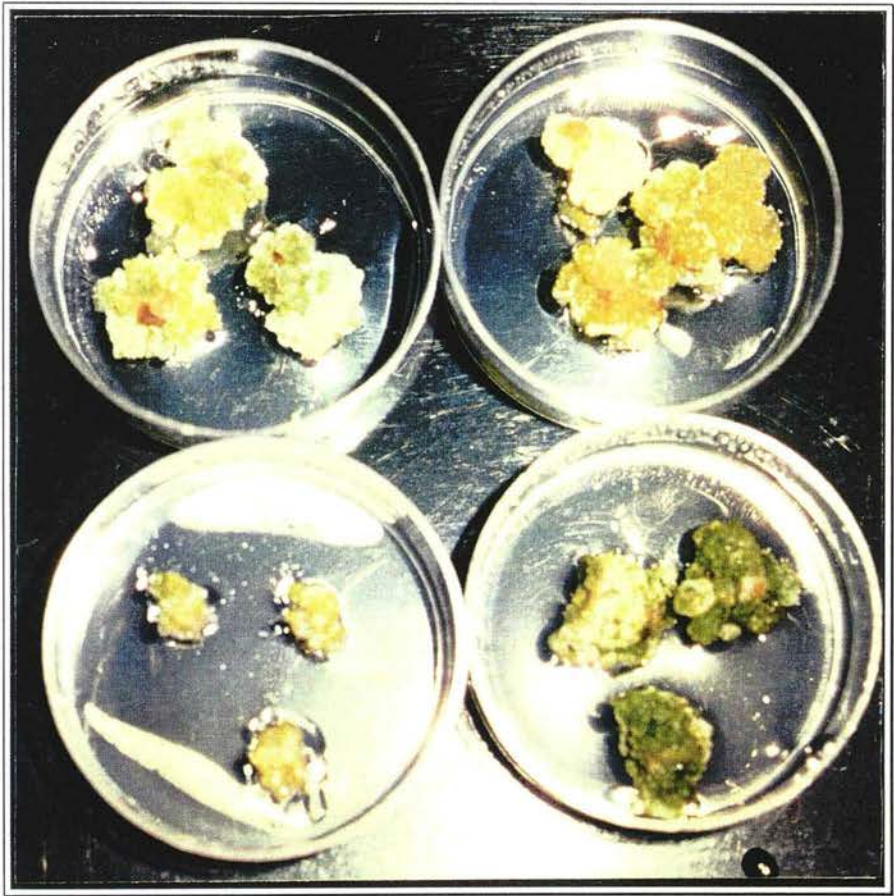
In all the experiments, except that in the 14th culture cycle, analysis of variance of relative fresh weight gain revealed highly significant ( $P<0.01$ ) or significant ( $P<0.05$ ) differences between the culture media, and the interaction between callus type and media (Appendix 4.17, 4.21 and 4.24). Effects of callus type observed on callus growth, were not significantly different ( $P\geq 0.05$ ). Since the effect of interaction, was significant, for further information, callus growth from different callus type was analysed separately.

Again, the statistical analysis from these experiment indicated similar results to previous experiment. On non-selective medium, analysis of variance of relative increase in callus fresh weight showed no significant differences among callus type ( $P\geq 0.05$ , Appendix 4.18, 4.22 and 4.25). These analyses indicated that selection has



not significantly affected the normal growth behaviour of callus. Fig 4.8(a), and 4.9(a,b) also shows that there was no statistically significant difference in the growth of two callus type on non-selective media.

Analysis of variance of relative increase in callus fresh weight, on selective medium, showed highly significant ( $P < 0.01$ ) differences among callus type (Appendix 4.19, 4.23 and 4.26). These results indicated that growth on normal culture medium was not effected by selection but the selection increased the tolerance of the callus therefore, significantly more growth was observed on the selective medium from the selected callus as compared with that on un-selected callus. In general tolerance index calculated for selected callus was more than that for un-selected callus (Plate 4.6.)



**Plate 4.6. Callus growth from selected and un-selected callus on selective and non-selective medium. Top row : growth on salt free medium, Bottom row: growth on the medium with 1% NaCl. Left: un-selected callus, Right: selected callus.**

In the 17th culture cycle, all the selected callus type showed significantly more salt tolerance as compared with un-selected callus. Average relative fresh weight gain on 9S+1M and 8S+2M was not significantly different from each other. Average relative fresh weight gain from 10S callus was maximum which was significantly different from that on control and 8S+2M but not significantly different with that on 9S+1M (Fig 4.9b). Tolerance index for 1% NaCl was maximum for 10S callus (79.68%) and minimum (19.48%) for the un-selected one. Tolerance index for 8S+2M and 9S+1M callus was 50.36% and 55.49% respectively. These results indicated that, although selected callus was more salt tolerant as compared with control callus, salt tolerance of the selected callus was reduced by culturing the selected callus on salt free medium. Reduction in enhanced salt tolerance appeared to be directly proportional to the duration of exposure to salt free medium.

Results achieved from the experiment in the 14th culture cycle were very unpredictable and it was very difficult to draw conclusions. Analysis of variance of relative fresh weight gain, revealed highly significant differences between culture media ( $P < 0.01$ ), significant differences between callus type and non-significant differences ( $P \geq 0.05$ ) of interaction between callus type and media (Appendix 4.20). These analysis indicated that selection may have changed the growth behaviour of callus. Fig 4.8(b) shows that the callus selected continuously for 6 generation showed maximum growth in both the cases i.e. on selective as well as non-selective medium. There were not much difference among tolerance index of all three types of callus.

In general, these results indicate that selection did not changed the growth behaviour of callus at normal culture medium (except in 14th culture cycle). Selected callus showed more salt tolerance as compared with the control, which might be the effect of selection. Selected callus showed significantly more salt tolerance as compared with the control, even after growing on salt free medium for few generations. A portion of the enhanced salt tolerance of the selected callus was lost due to exposure of selected callus to salt free medium.



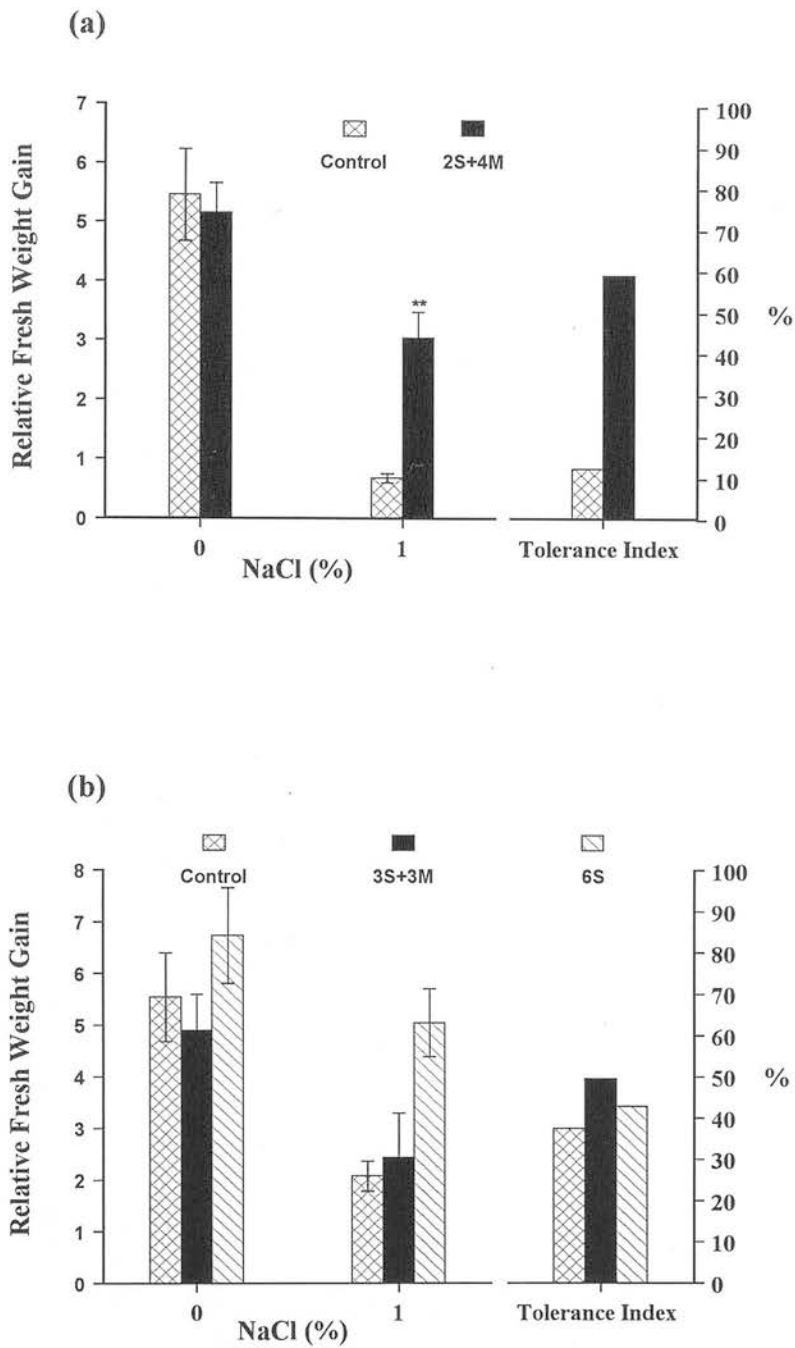


Fig. 4.9. Salt tolerance of different types of selected and un-selected callus of *Brassica rapa*. (a) stability test in 10th culture cycle (b) stability test in 14th culture cycle. Mean  $\pm$  SEM,  $n=5$ .

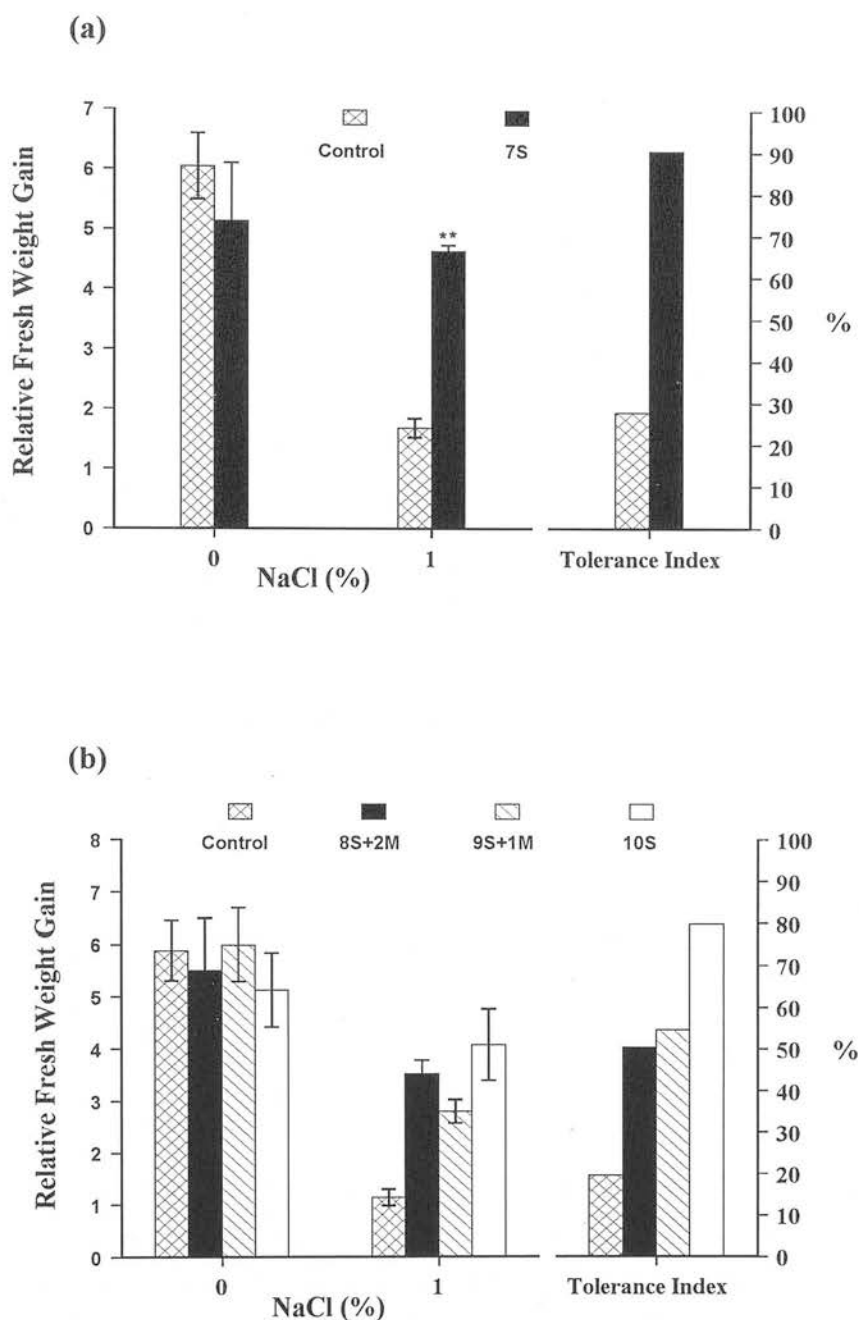


Fig. 4.10. Salt tolerance of different types of selected and un-selected callus of *Brassica rapa*. (a) stability test in 16th culture cycle (b) stability test in 17th culture cycle. \*\* Highly significant ( $P>0.01$ ). Mean  $\pm$  SEM,  $n=5$ .

## 4.5. Discussion

### Dose response curve

All soils contain a mixture of soluble salts, some of which are essential for growth. But salinity, defined simply as the presence of excessive concentration of soluble salts, (U.S. Salinity Laboratory Staff, 1954) suppresses plant growth. Such suppression of plant growth increases as the salt concentration increases (Bernstein and Hayward, 1958; Ashraf, 1994) until the plant dies. The same results were observed in the first part of this investigation. The effect of NaCl was studied on callus cultures, callus initiation and intact plants of different *Brassica* species. In all cases, in general, growth was decreased with increasing salt concentrations, ultimately resulting in death of plant material.

Selective concentrations of NaCl, for *in vitro* callus selection, were different for different *Brassica* species. In general, NaCl concentration of 1.25% or higher was found to be lethal for callus cultures of all the *Brassica* species studied. Selective concentrations for the species ranged from 0.75%-1.0% NaCl. Several reports also showed the selective concentration of NaCl for *in vitro* selection of *Brassica* species, to be in the same range. Jain *et al.* (1991) used 1% NaCl for the selection of cotyledons, callus and suspension cultures of *Brassica juncea*. 0.7% NaCl was used for the selection of protoplasts and callus of *Brassica napus* L. Raldugina *et al.* (1988) and Paek *et al.* (1988) selected shoot tips of chinese cabbage at 0.5% and 1% NaCl. These reports and the present finding indicate that NaCl concentration around 1% is suitable for the *in vitro* selection of *Brassica* species, but the exact concentration depends on the genotype under study and the *in vitro* culture system.

The effect of NaCl on callus initiation was determined by culturing hypocotyl segments, on culture media containing 0-1.75% (w/v) NaCl. Callus induction decreased with the increase in salt concentration. Jain *et al.* (1986) also observed reduction in callus initiation/growth with the increase of NaCl levels, from cotyledon explants of *Brassica juncea*. Generally, NaCl concentration >1.25% was lethal for callus initiation in all the species studied, under this project.

When seeds of three *Brassica* species were sown on different levels of NaCl, in general seedling growth was decreased with the increase of NaCl concentration and at a certain level no growth was observed. NaCl as a single salt affected the root length and shoot length negatively, resulting in decrease in seedling fresh weight.

Furthermore it was observed that a NaCl concentration more than 1.5% (w/v) was lethal for seedling growth of all the species under study. Different *Brassica* species showed differences in salt tolerance. Statistical analysis of seedling fresh weight in different *Brassica* species, indicated significant differences among *Brassica* species for NaCl tolerance. Differences among *Brassica* genotypes for salt tolerance has been well documented. Sinel'-Nikva and Boos (1983) studied 690 genotypes of different *Brassica* species for seed germination on different concentrations of NaCl and demonstrated differences between and within species. Similarly Rizk *et al.* (1979) found differences in NaCl tolerance of two varieties of rape. Rajasekaram and Shanmugavelu (1981) also observed NaCl tolerance differences between species and lines of genus *Brassica*. Sinel'-Nikova (1979) and Heerkloss and Bartolomaeus (1980) has also demonstrated differences in NaCl tolerance of different genotypes of *Brassica*.

Differences in salt tolerance of different *Brassica* species, were also observed in tissue cultures i.e. during callus induction and subsequent callus cultures. While studying the dose response curve through *in vitro* cultures, although the species under study were not compared statistically, the lethal and the selective NaCl concentration were found different for different species. Selective concentration for Curly kale and *Brassica rapa* was 1% NaCl and for winter oilseed rape it was 0.75%. NaCl concentration at 1.50% or more was lethal for callus cultures of most of the species of *Brassica*. Chandler *et al.* (1986) has also reported difference in sensitivity of different *Brassica* species to sodium sulphate in tissue culture. Similarly He and Cramer (1993) also observed differences in the callus of two rapid cycling *Brassica* species, for salt tolerance.

Although, investigations on the dose response curves, in different culture systems, indicated that lethal and selective NaCl concentrations were different in different culture systems, for all the species studied, the differences were small. For example, highest NaCl concentration allowing growth of intact plants, callus cultures and callus initiation were 1.5%, 1% and 1.25% in curly kale, 1.25%, 0.75% and 1% in *Brassica rapa* and 1.25%, 0.75% and 0.75% in winter oilseed rape respectively. These slight differences might be due to different cellular organisation in different material. In general intact plants showed slightly more salt tolerance (as measured by the lethal concentration) as compared with that *in vitro* cultures. The possible explanation of this increased tolerance shown by the intact plants, may be that many mechanisms are involved to deal with excess salts in soils. Some of these

mechanisms, by which plants show salt tolerance, utilise numerous cells and tissues in a co-ordinated series of processes in order to effect salinity tolerance. Therefore some processes may require an anatomical organisation (e.g. transport mechanisms, salt glands, specialised trichomes) which exist in intact plants. Other mechanisms by which plant deals with salinity, involves properties intrinsic to individual cells (Binzel *et al.*, 1985). In the present study the intact plants showed more salt tolerance as compared with callus or plant parts (hypocotyl), because intact plant has both type of mechanisms i.e. anatomical organisation and intracellular mechanism where as *in vitro* cultures cells by and large, have no anatomical cellular organisation. Another possible reason for this higher salt tolerance shown by intact plants as compared with that tissue cultured cells might be difference of osmotic potential in two solutions (i.e. NaCl+distilled water and NaCl+MS-medium+distilled water). The osmotic potential of NaCl solution with MS-medium was more, in all the concentrations, as compared with that without MS-medium. Therefore tolerance level of *in vitro* grown material may reflect intracellular control of salt in intact plants.

### ***In vitro* selection**

Callus from all the species under study, was selected on the selective concentration of NaCl, specific to the species. Generally, about 40-50% of the total callus produced on the control medium, was able to grow on the selective medium. Curly kale provided most growth on the appropriate selective medium, with more than 50% (of the callus growing on salt free medium, by weight) surviving and growing.

Many NaCl resistant cell lines have been isolated from considerable number of species (Dix, 1986; Dix *et al.*, 1986; Nabors, 1990; Tal, 1990). Successful *in vitro* selection for salt resistance in *Brassica* species has been reported by many researchers (Chandler *et al.*, 1986; Hodgkin, 1986; Chandler and Thrope, 1987; Paek *et al.*, 1988; Jain *et al.*, 1991; Kirti *et al.*, 1991a,b). Increased tolerance to different abiotic stresses, resulting from *in vitro* selection has also been reported in crop species other than *Brassica*. Woo *et al.* (1985) reported successful attempts by selecting rice callus with up to 1.5% NaCl. Jain *et al.* (1987) reported successful selection of eggplant cells. Binzel *et al.* (1985) had also reported successful selection in tobacco.

### **Stability tests**

Although, in most cases, *in vitro* selection has resulted in stable salt tolerance even after growing on salt free medium for many generations, there are still many

reports indicate that tolerance was not stable and was reversible when cells were transferred to a salt free medium. In this study the stability of the selected callus for salt tolerance was different for different species, studied. Out of three species studied, only one produced stable (but partially revisable) NaCl tolerant cell lines. The other two species (curly kale and winter oilseed rape) produced callus which showed more salt tolerance as compared with that of the control, however the differences were not significant statistically. Chandler *et al.* (1988b) also reported failure to successful *in vitro* selection with different *Brassica* species. Unsuccessful *in vitro* selection has been observed in other crop species as well. McCoy (1987a) selected few *Medicago* species tolerant to different levels of NaCl and reported that all, but one, of the genotypes that had demonstrated NaCl tolerant *in vitro* were NaCl sensitive at whole plant level. Bressan *et al.* (1985) found reversible as well as non-reversible NaCl tolerance from selected cell lines of tobacco. Chandler and Vasil (1984) selected callus of napier grass and no retention of tolerance was observed after the exposure of callus to salt free medium.

Although salt resistance, after *in vitro* selection was not found stable, for curly kale and winter oilseed rape, in all the studies it was observed that callus growing continuously on selective medium was more salt tolerant as compared with the control callus. Possible reasons for this increased tolerance have been reported by Hasegawa *et al.* (1980) and Binzel *et al.* (1985). According to these reports, when cultured tobacco cells are inoculated onto medium containing excessive NaCl they exhibit an increased lag period, but eventually begin growing if NaCl concentration is not too high. The cells which result from this period of growth, then are able to grow more rapidly when challenged with same level of NaCl a second time.

Generally stability test results for *Brassica rapa* indicated some significantly positive effects of selection resulting in stable tolerance. In most cases, in general, selection increased salt tolerance and that salt tolerance was stable, at least in tissue culture, even when the cultures were grown on non-selective medium for few generations. Jain *et al.* (1991) selected *Brassica juncea* callus at high salt concentrations and the resistance was found stable up to shoot regeneration. Kirti *et al.* (1991,ab) found stable resistance in the seed of the next generation after selection of somatic embryos of *Brassica juncea*. Many workers have also reported stable resistance from *in vitro* selection of different *Brassica* species (Pua and Thrope, 1986; Jain *et al.*, 1986; Chandler and Thrope, 1987). Stable salt tolerance after *in vitro* selection has also been reported in crop species other than *Brassica*, such as *Citrus*



*cinensis* (Ben-Hayyim and Kochba, 1983; Ben-Hayyim and Goffer, 1989), Flax (McHughen and Swartz, 1984), *Cicer arietinum* (Pandy and Ganapathy, 1984); *Solanum melogena* L. (Jain *et al.*, 1987). Beloualy and Bouharmont (1991) selected cell line of *Poncirus trifoliata* and the stability of resistance was observed even in the regenerated plants. Gulati and Jaiwal (1993) observed stable resistance to Trans-4-Hydroxy-1-Proline in case of *Vigna radiata* tolerant cell lines.

In general, from all the species it was observed that callus growing continually on selective medium showed more salt tolerance as compared with the selected callus which was maintained on salt free medium after selection. Two potential explanations could account for this increased tolerance. 1: Callus growing continuously on selective medium has passed more time under the selective conditions as compared with the callus selected and then maintained on salt free medium and increased selection duration may have resulted in increased salt tolerance. 2: Selected callus may have reversible salt tolerance, due to physiological adaptation, to the salt in the medium. When this callus was exposed to the salt free medium, it lost the property to tolerate. The later explanation could be supported by the study mentioned under Section 4.4.4. In the investigation carried out in 17th culture cycle of *Brassica rapa*, three different types of selected calli were studied. Selection duration in all cases was increased up to 8-10 generations, so that slight differences in selected duration has minimum effects on salt tolerance. Two types of the selected callus (8S+2M and 9S+1M), which were exposed to salt free medium after selection, showed significantly less salt tolerance as compared with the callus selected continuously (10S). Salt tolerance decreased even when the callus was exposed to salt free medium only for 1 culture cycle. Nevertheless all three types of the selected callus showed significantly more salt tolerance as compared with the non-selected callus. These results indicate that cells in the selected callus, showing enhanced salt tolerance as compared with control callus, have different properties i.e. stable for salt tolerance and/or reversible salt tolerance. The part of this increased tolerance lost, when the selected calli were exposed to the salt free medium, may be due to reversible salt tolerance or physiological adaptation to the selective medium. Generally, physiological adaptation works only until the cultures are in the specific conditions and the ability is lost when the conditions are removed. The other part of the salt tolerance which was stable even after the culture on salt free medium, was considered due to other changes in cell population (genetic and/or epigenetic). Physiological adaptation of callus was observed more in the case of curly kale and winter oilseed rape and less in *Brassica rapa*. These findings have been supported by many reports. Bressan *et al.* (1985)

have shown (reputedly for the first time) that increased tolerance to NaCl results from both reversible adaptation of all cells in the population and an enrichment of the population for stable, more tolerant cell types. It has been suggested (King, 1984; Dix *et al.*, 1984) that adaptation to salt stress plays a significant role in the selection of resistant lines, many of which do not results from genetic changes. Mandel *et al.* (1989) has also reported physiological adaptation of *Brassica napus* L. callus tolerant to sodium sulphate.

From these studies the following conclusions may be drawn (1) The selection increases salt tolerance which may be stable or unstable. The increase in tolerance may be due to physiological adaptation to stress or may be due to somaclonal changes (genetic or epigenetic) in cultured cells. During this project up to 90% tolerance index had been observed in selected callus while in case of un-selected callus it was not observed more than 60%.

(2)Although from the literature available and the results of the current studies it is clear that selection increases tolerance index in tissue culture, the selection may or may not be effective. Selection would be effective only if the increased tolerance is stable and especially if it is due to genetic changes. The conclusion drawn from these results is that stability of salt tolerance is specific to species. In this project, out of three species only one showed significant stable salt tolerance in tissue culture. The stability of tolerance was observed even after culturing the selected callus without salt for 4 generations. The other two genotypes also showed increased tolerance in selected callus as compared with un-selected, even after growing on non-selective medium for few generations. But the difference was not significant.

(3)Lastly, the increased tolerance shown by the selected groups of many cells may be due to different mechanisms acting alone or in combination. In practice stable resistance shown in tissue culture may be the result of genetic (or epigenetic) change and the unstable part of the resistance due to physiological adaptation of the callus to stress conditions.



## 5. SOMACLONAL VARIATION

### 5.1. Introduction

Crop improvement is dependent upon the introduction or initiation of genetic variability. Traditional sources of variation include pre-existing variation in the existing germplasm, introduction of new germplasm, mutation induction breeding, hybridisation, and utilisation of un-collected, un-acquired, un-dated or unevaluated germplasm. More recently somaclonal variation has been suggested as a possible additional source of variation. Somaclonal variation has been defined as the variation displayed amongst plants regenerated through tissue culture (Larkin and Scowcroft, 1981). Broadly speaking somaclonal variation includes any variation displayed amongst any type of cultured material (e.g. cells, tissue and regenerated shoots etc.). Observation of somaclonal variation has been well documented in many reports, for a number of crop species (Evans, 1989; Bajaj, 1990; George, 1993). Presence of somaclonal variation in *Brassica* has also been reported by many researchers (Horak *et al.*, 1975; Hoffman, 1978; Grout and Crisp, 1980; Wenzel, 1980; Jain *et al.*, 1989; Jain *et al.*, 1991)

Somaclonal variation probably reflects both pre-existing genetic differences and tissue culture induced changes. The variation arising through tissue culture may be genetic or epigenetic in nature or physiological changes resulting in altered development. Somaclonal variation has been observed for a number of important agronomic characters such as phytotoxins, herbicides, nematodes, viruses and salt tolerance, high yield, male sterility, protein, sugar, and oil content. Many important crops such as wheat, rice, maize, potato, sugarcane, *Brassica* etc. have already yielded positive results to the extent of new cultivars being released (Bajaj, 1990).

Among somaclonal variation the most widely observed variation is chromosomal aberration especially variations in chromosome number (Evans, 1986; Bajaj, 1990). Currently methods employed to assess the presence of somaclonal variation are: morphological studies (Shepard *et al.*, 1980; Jain *et al.*, 1991), protein electrophoresis (Scowcroft *et al.*, 1985; Eapen *et al.*, 1989; Jain *et al.*, 1989), progeny testing (Jain, 1991; Vamling, 1993), cytological studies (Heinz and Mee 1969; D' Amato, 1977) and DNA fingerprinting/profiling (Landsmann and Uhrig, 1985; Vallés *et al.*, 1993). Among the most commonly used studies are cytological

evaluations for chromosomal variations and phenotypic studies for the morphological characters.

Morphological studies and protein electrophoresis provide indirect information on genetic changes occurring via tissue culture. The limitations to the use of these techniques for such analysis include environmental interaction influencing expression of characters and the need for progeny testing to provide information of value for genetic analysis. Provided that these limitations can be satisfied such information can be used to derive some understanding of the nature of the genetic changes occurring in tissue culture. But progeny testing is a time consuming process. Direct analysis to investigate genetic changes, occurring in tissue culture, include cytological analysis and DNA profiling. Cytological analysis for chromosomal changes has already been used by many researchers for regenerated plants. These techniques identify structural changes in chromosomes but does not provide information on lesser changes in genetic make up. In addition chromosomal studies from callus is very difficult because all the cells in callus are not growing synchronously.

DNA profiling is the most recent approach for the detection of variation and the analysis is based on the genetic make up only. Techniques include RFLP and RAPD. Among the different techniques used for DNA profiling RAPD-PCR is a quick and easy technique to detect genetic variation. It is based on the total genomic DNA and reflects variation in genetic make up only. Although there are many limitations in this technique it still has many advantages over other techniques. These advantages has been presented in section 1.3.7.

As already mentioned in chapter 3, only one species i.e. curly kale was able to regenerate plants from callus. Therefore the study of somaclonal variation, in both callus and regenerated plants, was possible only in curly kale. Samples only from curly kale were subjected to RAPD analysis to study somaclonal variation. Tissues from different cultures including parent and regenerated plants, stored at -80°C (Section 2.11.) were used for this analysis.

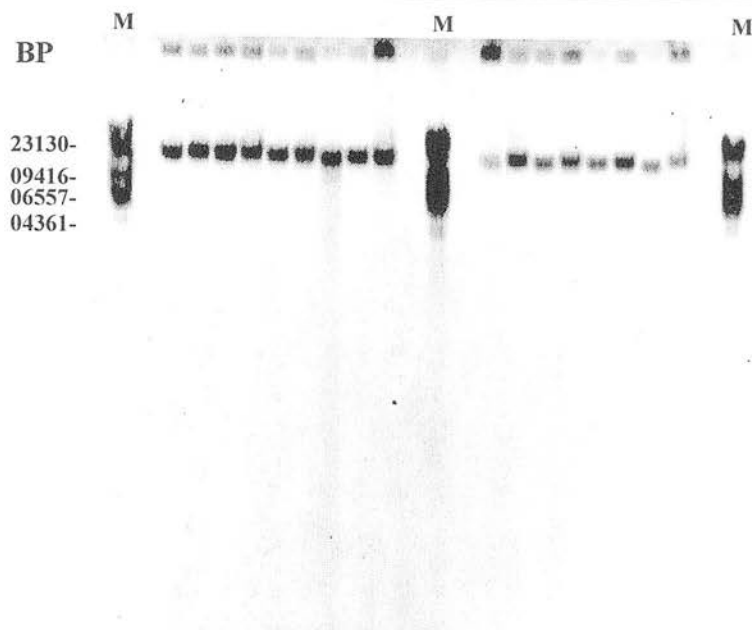
The aims of the current study were to detect and quantify the amount of somaclonal variation using RAPD analysis. To study DNA polymorphism related to salt tolerance and to investigate factors affecting the somaclonal variation.

## 5.2. DNA extraction

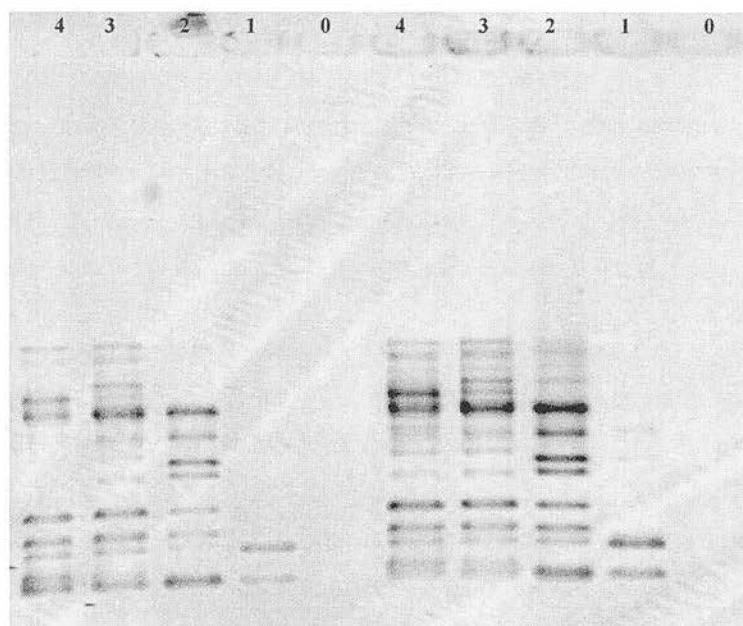
Extraction of DNA is the first step in RAPD-PCR analysis. One of the most appealing feature of the PCR is that the quality and quantity of the DNA to be subjected to amplification does not need to be high (Saiki, 1990), and the size of target DNA is generally not a critical factor (Sambrook *et al.*, 1989). According to these and many other reports, it is not absolutely necessary that the target DNA should be of very good quality for PCR reactions, however good quality DNA gives better and more reliable results (Higuchi, 1989).

The methods used for the extraction of DNA should be easy and quick and the DNA isolated should be of good quality with damaged as little as possible by the extraction method. Previously different methods have been used for the extraction of DNA (Murray and Thompson, 1980; Saghi-Marooof, 1984; Junghens and Metzloff, 1990; Liu *et al.*, 1990; Doyle and Doyle, 1987). In this study DNA was extracted by Pure Gene DNA isolation kit, following the procedure recommended by the manufacturer (Section 2.11.1). This DNA extraction method was found to be easy and quick and the DNA extracted was of good quality.

To confirm the successful extraction the extracted DNA was visualised after running in a 1.4% agarose gel and subsequently stained in ethidium bromide (Section 2.11.2). Extracted DNA showed a single, sharp band of about 23 K bp (Fig. 5.1). DNA of any sample showing smearing or no sharp band was discarded and the extraction was repeated. After the successful extraction the DNA, samples were quantified and qualified by using spectrophotometer (Section 2.11.3). The samples showed about 150-320  $\mu\text{g ml}^{-1}$  of DNA with  $\text{OD}_{280}/\text{OD}_{260}$  value ranging from 1.6 to 1.75. All the samples were diluted accordingly to make the final concentration 25  $\text{ng } \mu\text{l}^{-1}$ . Two  $\mu\text{l}$  of the DNA solution was used in each 50  $\mu\text{l}$  PCR reaction, as template.



**Fig. 5.1.** DNA extraction through Puregene™ DNA isolation kit. DNA was run in 1.4% agarose gel in TBE-buffer for 1½ hours and stained in ethidium bromide solution. M is for marker and BP for segment size of the DNA marker in base pairs. Other lanes are of DNA samples of curly kale.



**Fig. 5.2** Effect of different concentrations of  $\text{MgCl}_2$  on DNA amplifications using PCR (2 replications). Number of each lane represents the mM of  $\text{MgCl}_2$  used in PCR reaction.

### 5.3. RAPD-PCR analysis

The method referred to as Arbitrarily Primed (AP) or Random Amplified Polymorphic DNAs (RAPDs) is based on the Polymerase Chain Reaction (PCR) amplification of genomic DNA with a set of short primers of arbitrary sequence. Even in the absence of specific nucleotide sequence information, these primers can detect polymorphism in the form of variable number and length of PCR products synthesised in different species or individuals. The polymorphism function as genetic markers which can be used to detect differences between different individuals.

Mostly RAPD techniques have been used for the detection of similarities between different closely related genotypes, to establish relationships. Although there are only a few reports available for the use of RAPDs to detect somaclonal variation, in all the reports the techniques was found to be easy and useful for the detection of variation (Isabel, *et al.*, 1993; Vallés, 1993; Guzikirmizi *et al.*, 1993).

For Random Amplified Polymorphic DNA, the reactions were incubated in a GeneE thermocycler. The PCR protocol used was as described by Williams *et al.* (1990) with some modifications (Section 2.10.4.). Decamer oligonucleotide used as random primers were purchased from Operon Technologies Inc. These primers were from kit 'A' and kit 'D' (of 20 primers each) The nucleotide sequence of each primer was randomly generated with G+C content of 60-70%, with no self complementary ends. Oligonucleotides purchased were in the form of lyophilised powder. The primers were re-suspended in an appropriate quantity of sterile deionised water to make the final concentration 10  $\mu$ M. The primers solutions were divided in to several aliquots and stored at -20°C until used.

#### 5.3.1. Estimation of $MgCl_2$ quantity for PCR reactions

For best PCR results, optimisation of the different reagents used in PCR reaction mixture and other PCR conditions is a prerequisite. These include, quantification of target DNA, dNTPs, polymerase, primers,  $MgCl_2$ , temperature and duration of each PCR cycle and the number of total cycles for amplifications. Changes to the PCR buffer usually affects the outcome of amplifications. In particular, the concentration  $MgCl_2$  can have a profound effect on the specificity and yield of an amplification (Saiki, 1989). The magnesium ion concentration may affect all of the following: primer annealing, strand dissociation temperature of both template and PCR product, product specificity, formation of primer-dimer

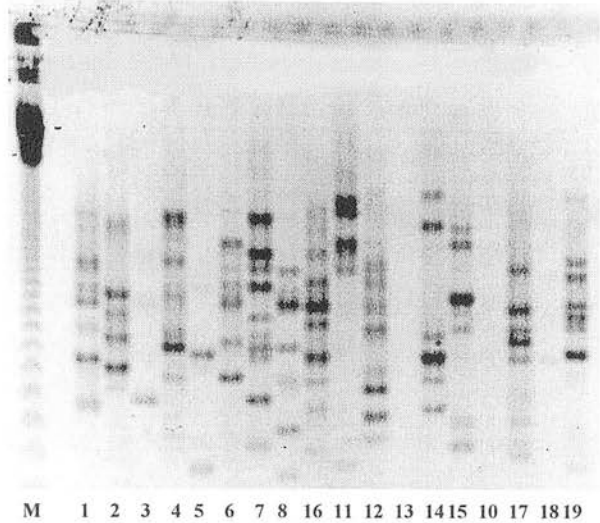
artefacts, and enzyme activity and fidelity (Innis and Gelfand, 1990). Therefore it is beneficial to optimise the magnesium ion concentration for any PCR programme.

In this study five different concentrations of the  $\text{MgCl}_2$  (i.e. 0, 1, 2, 3, and 4 mM) were examined keeping all other reagents constant. DNA of the parent plant was used as template and all other condition were the same for all  $\text{MgCl}_2$  treatments. The amplification was carried out in two replications. The PCR protocol followed was as described under Section 2.10.4.

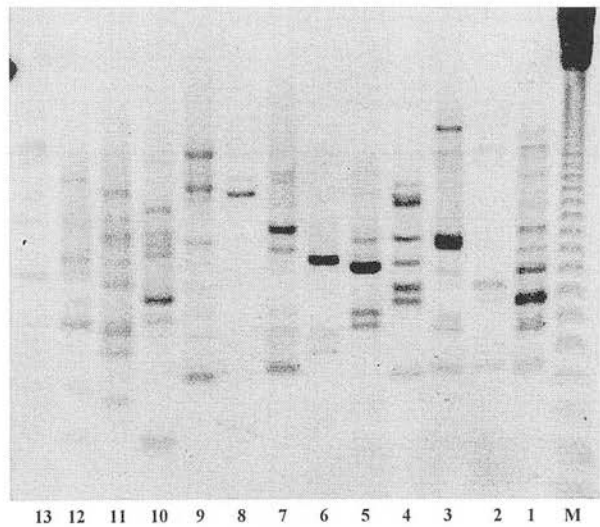
The results obtained were quite clear (Fig 5.2). 0 mM  $\text{MgCl}_2$  (Lane 1) did not showed any bands, while at 1 mM  $\text{MgCl}_2$  concentration (Lane 2) only two bands were visible.  $\text{MgCl}_2$  concentration from 2-4 mM showed more than 10 visible bands from the same primer and DNA sample. At 2 mM  $\text{MgCl}_2$  all the bands observed were very clear and sharp but a few of the bands were missing which were visible, if faint, at the concentration of 3 and 4 mM  $\text{MgCl}_2$ . At the concentration of 3 mM  $\text{MgCl}_2$  all the bands were visible and were very clear, while at 4 mM  $\text{MgCl}_2$ , although all the bands were visible, they were less sharp as compared with those at 2 or 3 mM  $\text{MgCl}_2$ . Therefore it was decide that 3 mM  $\text{MgCl}_2$  would be used for PCR reaction in this study.

### 5.3.2. Study for primers

For RAPD analysis, most primers are selected at random, without prior selection. Primers able to amplify more DNA fragments give more information and if more numbers of fragments are amplified there are greater chances of polymorphic fragments. Therefore the primers were tested using the DNA from parent plant as template. The numbers of random fragments amplified by each primer was counted and primers not showing satisfactory amplification were discarded from the programme. The primers showing many bands were preferred over primers showing few bands. A few of the primers showing many bands were later used to differentiate between samples. The bands produced by different primers of the two kits has been shown in Fig. 5.3 and 5.4.



**Fig 5.3.** DNA profiling using different arbitrary oligonucleotides of kit A. For all the primers the DNA used was from seed grown plants of curly kale. M represent 123 base pair ladder marker (Sigma). The number represents the code No. of the primers in kit A (OPA-#)



**Fig 5.4.** DNA profiling using different arbitrary oligonucleotides of kit D. For all the primers the DNA used was from seed grown plants of curly kale. M represent 123 base pair ladder marker (Sigma). The number represents the code No. of the primers in kit D (OPD-#)



### 5.3.3. Variations in cultures

DNA was extracted from different samples including the parent plant, regenerated from seeds. Different samples were collected at different times depending on their availability. DNA was extracted in groups of samples which were available at the time of extraction. These samples were collected from three types of material, i.e. regenerated plants which were transferred to the glasshouse and were growing, from regenerated shoots which were still under *in vitro* conditions and callus of different culture cycles.

A total of 10 primers were used for analysis in this investigation. Total number of primers studied for different samples were different because the samples which were collected earlier were subjected to more PCR reactions as compared with the samples which were collected later, and in different reactions some samples did not show usable results which was supposed due to pipetting error. Those samples were excluded from the analysis for that specific primer. The primer code and their sequences, provided by the supplier, are shown in Table 5.1. The numbers of primers used for analysis of each sample are shown in Table 5.2.

**Table 5.1.** Base sequences and molecular weight of different decamer oligonucleotides from kit 'A' and 'D' (Operon Technologies Inc.) used for the detection of somaclonal variation in curly kale.

Code	Sequence 5' to 3'	Molecular weight
<b>KIT A</b>		
OPA-02	CAGGCCCTTC	2955
OPA-06	GGTCCCTGAC	2995
OPA-07	GAAACGGGTG	3108
OPA-09	GGGTAACGCC	3044
OPA-17	GACCGCTTGT	3010
<b>KIT D</b>		
OPD-04	TCTGGTGAGG	3090
OPD-09	CTCTGGAGAC	3019
OPD-10	GGTCTACACC	2979
OPD-13	GGGGTGACGA	3124
OPD-20	ACCCGGTCAC	2964

In each PCR, DNA from parent plant (grown from seeds of original lot) was also included and each sample was compared with parent plant for differences. Each reaction was repeated with the same conditions, and only the reproducible bands were included in the assay. Faint bands which were difficult to observe were also disregarded in the assay. Therefore, possibly additional useful information was omitted to ensure the reliability of the assay. A negative control (without target DNA) was always run in each PCR amplification, to detect any contamination in the reaction mixture. Amplification in the negative control was considered a contamination in any of the reagents in reaction mixture.

Amplification products of each DNA fragment, were represented by a band on a gel/photograph. Each band represented a set of DNA segments/fragments of almost equal length. Therefore, the words 'segment', 'fragment' and 'band' has been used synonymously, representing a single set of DNA segments of equal length. The number of bands produced by each primer for each sample was scored. Total number of bands produced by all the primers used for a single sample were calculated and compared with total number of bands produced by the same primers for the parent. Data generated from detection of the fragments were analysed employing the equation of Nei and Li (1979) (Section 2.10.4), and similarity between parent and individual sample was calculated. Variation between parent and each sample was calculated as below.

$$\text{Variation}(\%) = (1 - \text{Similarity}) \times 100$$

Variation of different samples to parent has been shown in Table 5.2. The table shows that variation between parent and cultured material (samples) ranges 0.62% to 11.11% only. Looking at the results of individual primer, in some cases variation was too obvious to be neglected, although its fraction was very small. The most prominent variations shown by specific primers will be discussed here taking individual primers one by one. Results of some important PCR-reactions have been illustrated in Fig 5.5 to Fig. 5.13. The name of the primers (as code No.) used here are the same as given by the supplier (Table 5.1).

**Table 5.2. Variation in different *in vitro* cultures of curly kale, estimated by RAPD-PCR amplifications.**

No.	Sample	Primers	Variation (%)
1	C3 (i), regenerated, transferred	6	1.54
2	C3 (ii), regenerated, transferred	6	4.12
3	C1, regenerated shoots	6	6.06
4	C2, regenerated shoots	7	5.56
5	C3 (i) regenerated shoots	7	5.56
6	C3,(ii) regenerated shoot	7	5.56
7	C3, (iii) regenerated, transferred.	7	8.57
8	C3, (iv) regenerated shoots	5	8.69
9	C2, regenerated, transferred	6	11.11
10	C3, (v) regenerated shoot	5	3.57
11	C3 (i), callus	6	2.94
12	C3 (ii), callus	6	1.64
13	C5, regenerated, transferred, plant 1	9	1.84
14	C5, regenerated, transferred, plant 2	9	6.49
15	C6, regenerated, transferred	9	0.62
16	C6, callus	7	2.78
17	C7, callus	7	2.70
18	C7, callus (2S+3M)	6	1.45
19	C6, regenerated, transferred, plant 2	5	1.82
20	C8, callus, i	5	5.66
21	C8, callus, ii (from No. 17)	5	7.32
22	C9, callus	6	7.69
23	C9, regenerated (2S+3M)	6	7.41
24	C9, regenerated, transferred, plant i	5	1.96
25	C9, selected and regeneration under selective conditions	6	5.08
26	C8, regenerated, transferred, plant i	10	2.22
27	C8, regenerated, transferred, plant ii	10	4.17
28	C9, regenerated, transferred, plant ii	5	1.82
29	C7(i), regenerated, transferred, plant	10	3.16
30	C7(ii), regenerated, transferred, plant	10	4.43
31	C10, regenerated shoot	6	4.88
32	C10, regenerated shoot (2S+4M)	6	1.59

Continue to next page

33	C10, regenerated shoot (4S+3M)	6	3.45
34	C10, regenerated (R2), transferred,	10	1.54
35	C10(i), regenerated (R5), transferred	13	3.87
36	C13, (6S)	6	3.70
37	C10(ii), regenerated(R5), transferred	10	3.13
38	C7(iii), regenerated, transferred	9	1.66
39	C8, selected, regenerated in salt	5	5.45
40	C8, regenerated, control	5	5.66
41	C15, (4S)	6	8.33

C culture cycles

S selection cycles

M maintenance on non-selective conditions after selection

R regeneration cycles

i,ii,iii... No. of plants or callus samples from the same age group

#### 5.3.4. RAPD profile by the primer OPA-06

Decamer oligonucleotide OPA-06 was used for DNA profiling of 9 samples including the parent line. All the DNA samples were from the regenerated and glasshouse transferred plants. A total of 4 bands were scored for the analysis. Out of these four fragments only one was found polymorphic and only one sample showed obvious polymorphism to the parent and other samples. Two prominent fragments between 2027-1904 bp range, were very close together, in the Fig. 5.6 these bands appear to be represented by a single bold band. The smaller one of these two DNA fragments was present in all the samples except in the DNA from C8 (i) plant (Fig 5.6, sample No. 2 on the gel). The rest of the DNA fragments were monomorphic in all the samples tested. All the samples showed 100% similarity to the parent and with each other, for these DNA fragments.

#### 5.3.5. RAPD profile by the primer OPA-07

DNA profiling from OPA-07 was carried out in two different PCR reactions using different samples. In the first reaction DNA from 13 different samples was amplified using this primer (Fig. 5.8). All the samples were from regenerated and glasshouse transferred plants. A total 12 DNA fragments were scored for the analysis. Four of these 12 DNA fragments were found polymorphic. All the 12 DNA fragments were present in the DNA profile of parent. A DNA fragment of slightly less than 1375 bp was missing from most of the samples. This fragment was missing from the DNA samples of C7 (i), (ii), (iii), C10 (i) and (ii) and C5 (ii) plants (Samples 5, 6, 7, 8, 9, and 12 respectively, in Fig 5.8). Another DNA fragment slightly less than 947 bp, which was quite obvious in all the samples, but was missing from the DNA samples of C8 (ii) plant only.

In the second reaction 32 DNA samples were amplified. A total 9 easily detectable DNA fragments were scored for the analysis. Four of these fragments were found to be polymorphic. Visual intensity of these polymorphic bands was low, therefore there were more chances of experimental error. Most of the sample showed polymorphism for these fragments (Fig. 5.7).

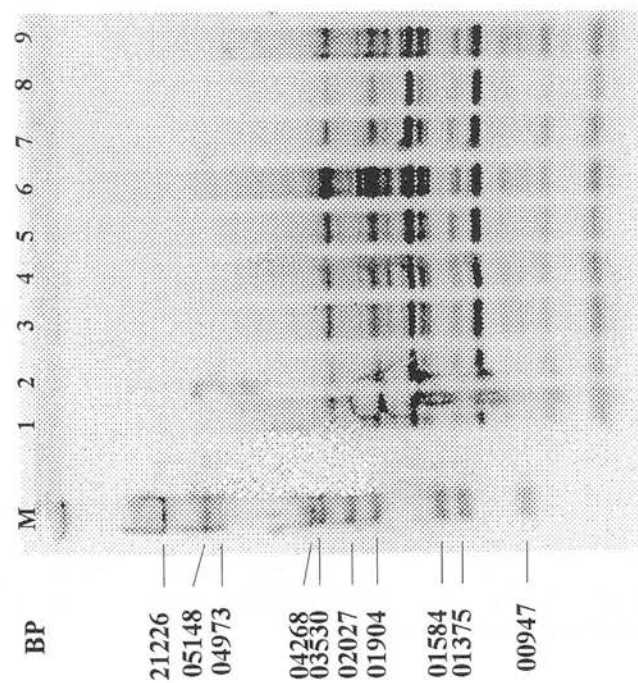


Fig. 5.5. RAPD profiles of 9 DNA samples generated by the primer OPA-02. M represents nucleic acid marker i.e. Lambda DNA, EcoRI Hind III digested. BP represents fragment sizes of marker DNA in base pairs. All DNA samples were from regenerated and green house transferred plants. Samples from left to right: 1- Parent (i), 2- parent (ii), 3- Parent (iii), 4- C5 (i), 5- C5 (ii), 6- C5 (iii), 7- C6, 8- C8 (i) and 9- C8 (ii)

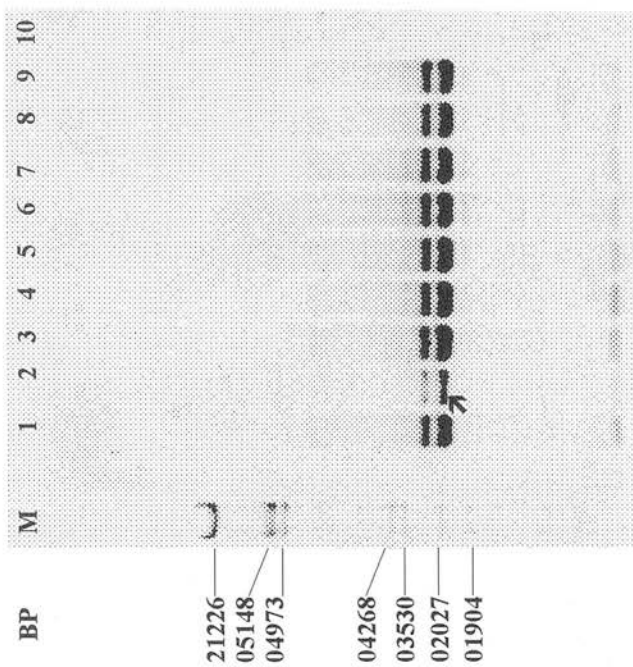


Fig. 5.6. RAPD profiles of 9 DNA samples generated by the primer OPA-06. M represents nucleic acid marker i.e. Lambda DNA, EcoRI Hind III digested. BP represents fragment sizes of marker DNA in base pairs. All DNA samples were from regenerated and green house transferred plants. Samples from left to right: 1- Parent (i), 2- C8 (i), 3- C8 (ii), 4- C10 (2 R), 5- C7 (i), 6- C57 (ii), 7- C7 (iii), 8- C10 (5 R, i), 9- C10 (5 R, ii) and 10- -ve control

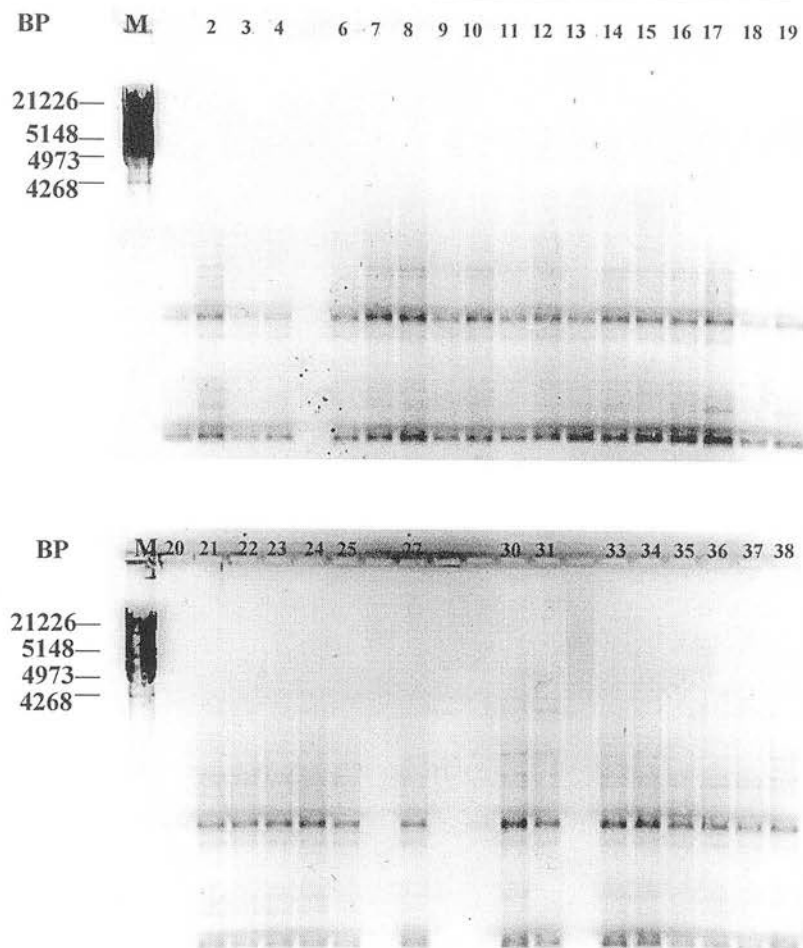


Fig. 5.7. RAPD profiles of 38 DNA samples generated by the primer OPA-07. M represents nucleic acid marker i.e. Lambda DNA, EcoRI Hind III digested. BP represents fragment sizes of marker DNA in base pairs. The DNA samples were from callus, regenerated shoots and regenerated and green house transferred plants. Samples from left to right:

2- Parent(ii)	11- C3(RT, iii)	19- C6(RT)	30- C8(RT, i)
3- Parent(iii)	12- C3(R, iv)	20- C6	31- C8(RT, ii)
4- C5(RT, iii)	13- C2(RT)	21- C7	33- C7 (RT, i)
6- C3(RT, ii)	14- C3(R, v)	22- C7(2S+3M)	34- C7 (RT, ii)
7- C1(R)	15- C3 (i)	23- C6(RT, ii)	35- C10 (R)
8- C2( R)	16- C3(ii)	24- C8	36-C10(R, 2S+4M)
9- C3(R, i)	17- C5(RT, i)	25- C8(ii)	37-C10(R, 4S+3M)
10- C3(R, ii)	18- C5(RT, ii)	27- C9(2S+3M)	38- C10(R2)

**Note:-** C: is for culture cycles, R: regenerated, T: green house transferred, S: selection cycles, M: maintenance cycles after selection and only C with out R or T, represents samples collected from callus



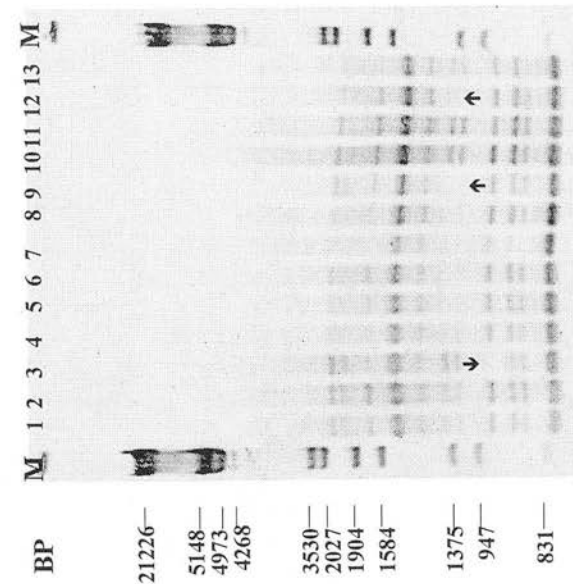


Fig. 5.8. RAPD profiles of 13 DNA samples generated by the primer OPA-07. M represents nucleic acid marker i.e. Lambda DNA, EcoRI Hind III digested. BP represents fragment sizes of the DNA marker in base pairs. All the DNA samples were from regenerated and greenhouse transferred plants. Samples from left to right: 1- Parent, 2- C8 (i), 3- C8 (ii), 4- C10 (2R), 5- C7 (i), 6- C7 (ii), 7- C7 (iii), 8- C10 (5R, i), 9- C10 (5R, ii), 10- parent (ii), 11- C5 (i), 12- C5 (ii) and 13 C6.

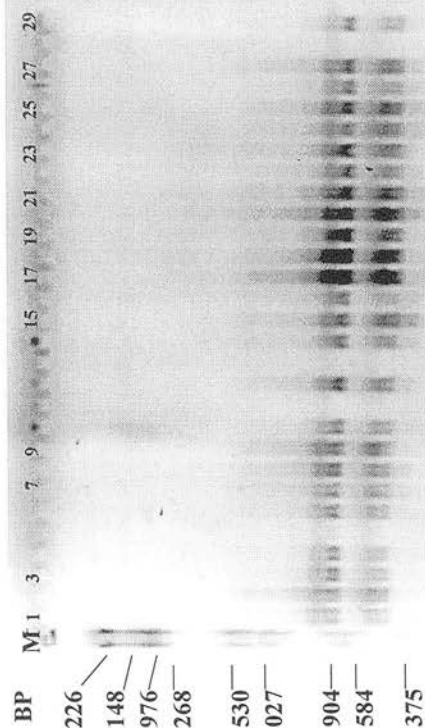


Fig. 5.9. RAPD profiles of 29 DNA samples generated by the primer OPA-09. M represents nucleic acid marker i.e. Lambda DNA, EcoRI Hind III digested. BP represents fragment sizes of the DNA marker in base pairs. The DNA samples were from callus, regenerated shoots and regenerated and greenhouse transferred plants. Samples from left to right: 1- Parent(i), 2- Parent(ii), 3- Parent(iii), 4- C5(RT, iii), 6- C3(RT, ii), 7- C1(R), 8- C2(R), 9- C3(R, i), 10- C3(R, ii), 12- C3(R, iv), 14- C3(R, v), 15- C3 (i), 16- C3(ii), 17- C5(RT, i), 18- C5(RT, ii), 19- C6(RT), 20- C6, 21- C7, 22- C7(2S+3M), 23- C6(RT, ii), 24- C8, 25- C8(ii), 26- C9, 27- C9(2S+3M), 29- C8(RT, i) and -ve control.

**Note** (Fig. 5.9):- C: is for culture cycles, R: regenerated, T: green house transferred, M: maintenance cycles after selection and only C with out R or T, represents samples collected from callus

### 5.3.6. RAPD profile by the primer OPD-09

DNA from 47 samples were amplified using primer OPD-09. A total of 6 DNA fragments were scored for analysis, two of which <947 bp were observed to be polymorphic. The clear polymorphism was shown only by three samples. A DNA fragment slightly more than 831 bp was missing in the regenerated and transferred plant of C8(ii) and regenerated shoots under selective conditions of C9 (sample 25 and 29, Fig. 5.10). While, another DNA fragment < 831 bp was also missing from regenerated and transferred plant of C8(ii), regenerated shoots under selective conditions of C9 and regenerated and transferred plant of C9(ii) (sample 25, 29 and 32, Fig 5.10). Other DNA fragments and the samples did not show any obvious differences.

### 5.3.7. RAPD profile by the primer OPD-10

DNA fragments produced by PCR reaction, using arbitrary primer OPD-10 have been shown in Fig 5.11. Nine DNA samples including the parent were amplified. All the samples were from regenerated and glasshouse transferred plants. A total 8 bands representing DNA fragments  $\leq 3530$  bp were scored to differentiate between different samples. Out of these 8, 3 DNA fragments were found to be polymorphic and only one sample, i.e. C8(ii) plant, showed polymorphism. All other samples were showing monomorphism with regard to these 8 DNA fragments. A DNA fragment of about 3530 bp, present in all other samples was missing in C8(ii) plant (Fig 5.11). An other DNA fragment slightly more than 1375 bp was also missing in this sample, which was replaced by two fragments very close to each other, at about the same position (Fig 5.11). All other samples and other fragments showed no polymorphism.

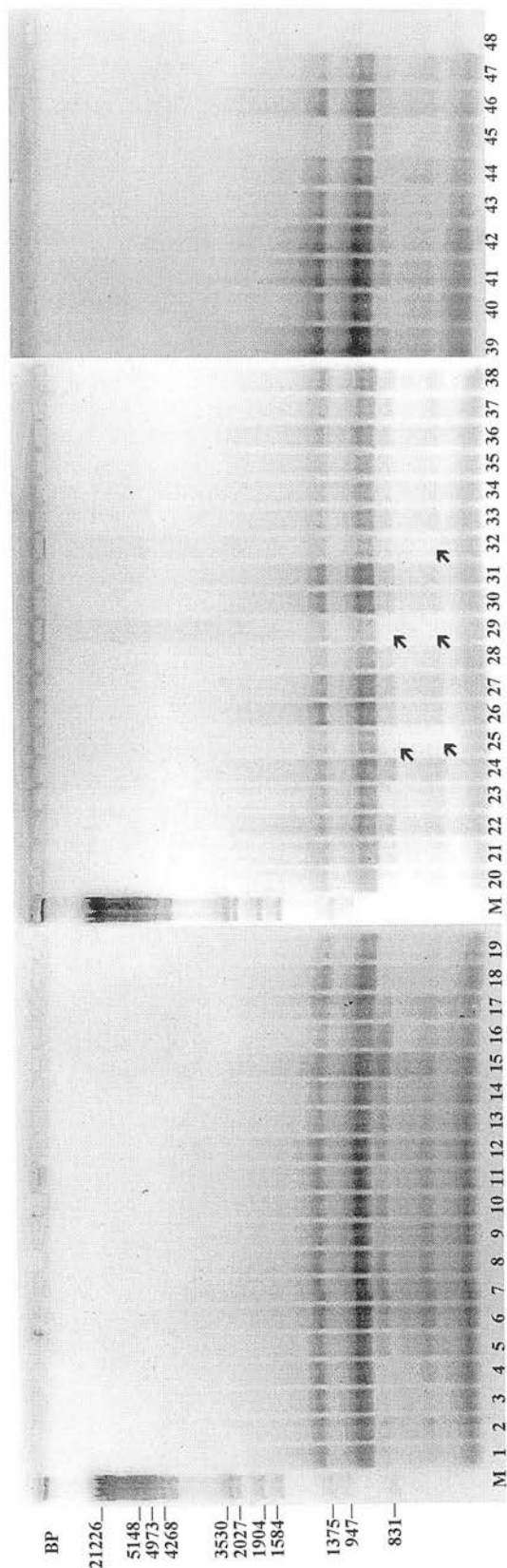


Fig. 5.10. RAPD profiles different DNA samples generated by the primer OPD-09. M represents nucleic acid marker i.e. Lambda DNA, EcoRI Hind III digested. BP represents fragment sizes of the DNA marker, in base pairs. The DNA samples from left to right:

1- Parent(i)	9- C3(R, i)	17- C5(RT, i)	25- C8(ii)	33- C7 (RT-i)	41- C10 (R5,ii)
2- Parent(ii)	10- C3(R, ii)	18- C5(RT, ii)	26- C9	34- C7 (RT-ii)	42- C7 (R, T, iii)
3- Parent(iii)	11- C3(RT, iii)	19- C6(RT)	27- C9(2S+3M)	35- C10 (R)	43- C8 (R in salt)
4- C5(RT, iii)	12- C3(R, iv)	20- C6	28- C9 (RT-i)	36- C10(R, 2S+4M)	44- C8 (R, cont)
5- C3(RT, i)	13- C3(RT, iii)	21- C7	29- C9(R under salt)	37- C10(R, 4S+3M)	45- C15 (4S)
6- C3(RT, ii)	14- C3(R, v)	22- C7(2S+3M)	30- C8(RT, i)	38- C10(R2)	48- -ve control
7- C1(R)	15- C3 (i)	23- C6(RT, ii)	31- C8(RT, ii)	39- C10 (R5, i)	
8- C2(R)	16- C3(ii)	24- C8	32- C9 (RT-ii)	40- C13 (6S)	

Note:- C: is for culture cycles, R: regenerated, T: green house transferred, S: selection cycles, M: maintenance cycles after selection and only C with out R or T, represents samples collected from callus.

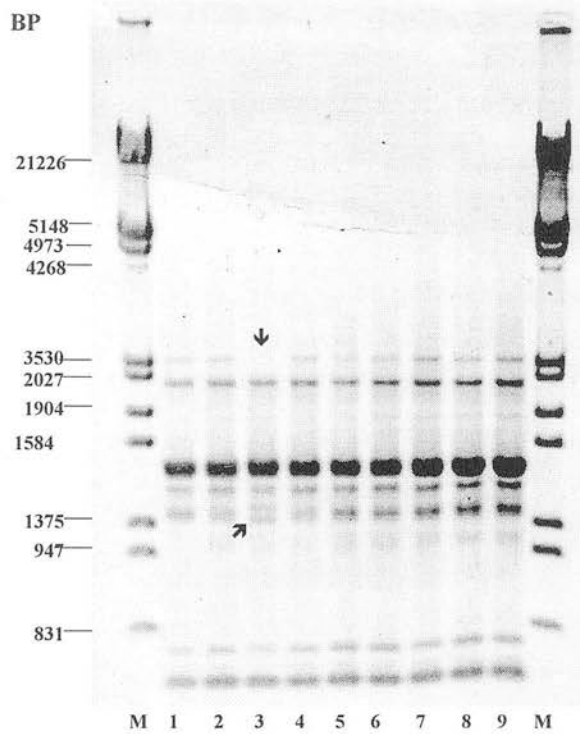


Fig. 5.11. RAPD profiles of 9 DNA samples generated by the primer OPD-10. M represents nucleic acid marker i.e. Lambda DNA, EcoRI Hind III digested. BP represents fragment sizes of the DNA marker in base pairs. All the DNA samples were from regenerated and green house transferred plants. Samples from left to right: 1- Parent, 2- C8 (i), 3- C8 (ii), 4- C10 (2R), 5- C7 (i), 6- C7 (ii), 7- C7 (iii), 8- C10 (5R, i) and 9- C10 (5R, ii).

### 5.3.8. RAPD profile by the primer OPD-13

RAPD profile by the primer OPD-13 was generated in two different PCR reactions and has been shown in Fig. 5.12 and 5.13. In the first reaction 9 DNA samples including parent plant were amplified. All the samples were from regenerated and glasshouse transferred plants. DNA fragments amplified by this primer showed obvious variation among the DNA from different samples. A total of 13 prominent and easily countable bands were used in analysis. Out of these 13 DNA fragments, 3 (between 1375bp-3530bp) were found to be polymorphic and were absent from few samples. The regenerated and transferred plant C8-ii (lane 3), showed the most prominent differences. The DNA fragment of about 831 bp was present in all the samples except from C8(ii) plant. A second prominent difference was observed in the DNA sample from the plant C7-i (lane 5 in fig 5.12). Two DNA fragments of size between 1904-2027 bp were missing in this sample, which were present in rest of all the samples. These differences are indicated by arrow head in Fig (5.12).

In the second PCR reaction DNA of 19 samples including the parent was amplified. A total 4 DNA fragments were found to be polymorphic in this reaction. The most prominent difference which was observed in the previous reaction, sample 3, was also observed in this reaction, sample 4 Fig 5.13. Another obvious difference was observed in DNA from C3(ii) callus (sample 16). A major DNA fragment of about 1375 bp was missing in this sample which was present in all other samples (fig 5.13).

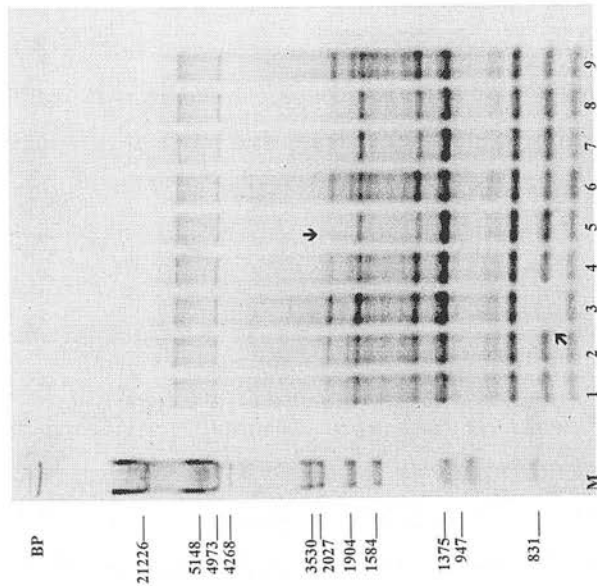


Fig. 5.12. RAPD profiles of 9 DNA samples generated by the primer OPD-13. M represents nucleic acid marker i.e. Lambda DNA, EcoRI Hind III digested. BP represents fragment sizes of the DNA marker in base pairs. All the DNA samples were from regenerated and green house transferred plants. Samples from left to right: 1- Parent, 2- C8 (i), 3- C8 (ii), 4- C10 (2R), 5- C7 (i), 6- C7 (ii), 7- C7 (iii), 8- C10 (5R, i), 9- C10 (5R, ii) and -ve control.

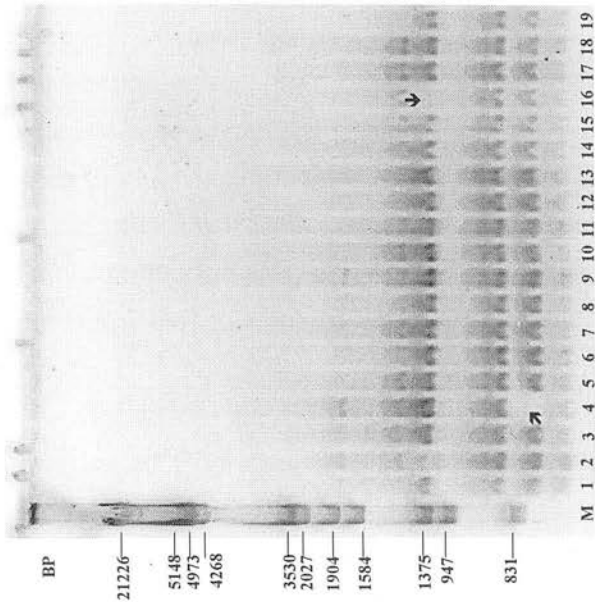


Fig. 5.13. RAPD profiles of 29 DNA samples generated by the primer OPD-13. M represents nucleic acid marker i.e. Lambda DNA, EcoRI Hind III digested. BP represents fragment sizes of the DNA marker in base pairs. The DNA samples were from callus, regenerated shoots and regenerated and green house transferred plants. Samples from left to right: 1- Parent(i), 2- Parent(ii), 3- C8 (i), 4- C8 (ii), 5- C10 (2R), 6- C7 (i), 7- C7 (ii), 8- C7 (iii), 9- C10 (5R, i), 10- C10 (5R, ii), 11- C5(i), 12- C5(ii), 13- C6, 14- C9, 15- C3 (i), 16- C3(ii), 17- C3 (iii), 18- C8 (R in salt), 19- C8 (control)

### 5.3.9. Presence of chimera in tissue culture

By definition a chimera is a plant or organism composed of tissues of two or more types (usually genetically different), that are capable of congruent and integrated existence (George, 1993; Leshem, 1990). This definition can be expanded to cover chimeric development in tissue culture i.e. callus may be composed of two or more types of cell which are genetically different with each other (this does not necessarily indicate that such cells operate in a congruent and integrated manner). The same definition for chimeras, in tissue culture, has also used by many workers such (Collin and Dix, 1990).

To study the existence of chimera in the present studies, different plants regenerated from the same callus piece were compared using the genetic markers derived by RAPD-PCR. If difference occurred between such plants then it would appear that the callus source contained more than one type of cells at the point of regeneration. In this project variation was also observed among different plants regenerated from same callus pieces. The variation to the parent shown by different regenerated plants from the same source, were different (Table 5.2). Presence of chimera was observed in many cases (e.g. regenerated and transferred plants in C5, C8, C9 and C10), the difference observed in derived plants of C8 culture are discussed below in more detail, as an example of the type of variation observed.

Two plants were regenerated from the same callus piece of C8 culture (C8-i and C8-ii) and transferred to the glasshouse after successful rooting. DNA profiles of these two plants, using arbitrary oligonucleotides showed clear polymorphism. When DNA from these two plants were amplified using OPA-06 primer, a DNA fragment of about 2027 bp was present in the DNA profiles from plant C8-ii, while the same fragment was missing from the profiles of C8-i plant (Fig. 5.6). DNA profiles using OPA-07, also showed a missing DNA fragment of slightly more than 947 bp, from the same plant (Fig. 5.8).

DNA polymorphism in these two plants, was also clear when primer OPD-10 was used for DNA profiling. DNA fragment of about 3530 bp which was present in C8-i plant was missing for C8-ii plant. An other DNA fragment of slightly more than 1375 bp was present in DNA profiles of C8-i plant while in the same region this fragment was replaced by two different fragments (Fig 5.11).



Genetic differences between these two plants were also very clear when OPD-13 primer was used for DNA amplification through PCR. Two clearly stained DNA fragments of about 1904 bp and 831 bp were present in plant C8-ii while the both fragments were missing from the DNA finger prints of C8-i plant (Fig. 5.12). These results confirmed the existence of chimera in tissue cultures especially in callus cultures.

#### **5.3.10. Genetic differences between selected and un-selected cultured material**

DNA from different phases of selected material was isolated and amplified using arbitrary oligonucleotides. Control material from the same cultures was always run with selected material. The samples were from C7 callus (sample 17, and 18, in Table 5.2), callus and regenerated shoots of C9 (sample 22, 23, 24, and 25), regenerated shoots of C10 (sample 31, and 32) and regenerated shoots of C8 (sample 39 and 40).

Although, the samples from the selected callus source showed variation (from 1.45%-7.4%) to the parent line (Table 5.2.), the variation observed could not be related specifically to the selection for salt tolerance. Examining the DNA profile in these samples there was not any specific remarkable polymorphism between selected and un-selected material, which could be related to salt tolerance.

These results indicated that no specific genetic marker, related to the NaCl selection, could be detected by the RAPDs in this study. Although there was some variation among selected and un-selected material, this variation does not appear to be related to NaCl tolerance. However it should be pointed out that sample size in this case was very small as was the number of primers examined. Or it may be due to the reason that no stable salt tolerance was observed in case of curly kale (Section 4.4.2).

#### **5.3.11. Effect of culture duration on somaclonal variation.**

For DNA amplification, cultured material was sampled from different cultures of different age i.e. from 1st (C1) to 13th (C13) culture cycles. Phases of the material was also different i.e. callus, regenerated shoots, regenerated and transferred plants.

Variation to parent, shown by different samples was not increasing or decreasing with respect to the change in culture period and/or number of culture cycles. Maximum variation was observed in one plant of C2 cycle (11.11%) while the regenerated shoots from the same number of culture cycles showed only 5.56%

variation to parent. The same was the case for other samples, and no effect of culture duration was observed on the variation. Therefore it is suggested that variation in tissue culture of curly kale was mainly by chance and regardless of the cultured period

## 5.4. Discussion

The DNA extracted from different samples showed a single, sharp band of about 23 kb, on agarose gel. A single sharp band, of high molecular weight, without smearing, indicates a good quality DNA with uniform segment size and less damaged during extraction process. Pure preparation of DNA have  $OD_{260}/OD_{280}$  values of 1.8 (Sambrook *et al.*, 1989). In this study the extracted DNA showed  $OD_{260}/OD_{280}$  value ranging from 1.6-1.75, which was slightly less than that of pure quality DNA. When the extracted DNA samples were tested for PCR work, the amplification was successful. Therefore the quality of the extracted DNA, using Puregene DNA Isolation kit, was considered satisfactory for PCR amplifications.

The optimum concentration of  $MgCl_2$  for PCR reaction was found to be 3 mM. The  $MgCl_2$  concentration used previously for PCR based amplification in *Brassica* species was different with different workers. Thorman *et al.* (1994) used only 1 mM of  $MgCl_2$  for RAPD-PCR reactions in cruciferous species. Santos *et al.* (1994) and Kresovich *et al.* (1992) used 2 mM  $MgCl_2$  for PCR, while Jain *et al.* (1994) used 2.5 mM  $MgCl_2$ . These reports indicated that suitable  $MgCl_2$  concentration may vary from reaction to reaction. Possible reasons for these differences might be differences in plant material studied, the quantity of other reagents used in PCR reaction or different PCR conditions.

Before analysing the samples, the primer from two kits i.e. A and D (Operon) were tested using DNA from a single plant, grown from the seed, as target DNA. Most of the primers tested showed satisfactory results. Satisfactory results from most of the primers indicated that the amount of different reagents in the PCR reactions mixture and the PCR protocol used was appropriate for these primers.

Although some of the primers showed quite obvious variation in a few of the samples, the variation shown by different samples to the parent line was variable in the samples (1-11.11% variation to the parent). Previous reports on the somaclonal variation are also variable. Isabel *et al.*, (1993) used RAPDs to evaluate the genetic integrity of somatic embryo genesis-derived population of *Picea mariana* and found no variation within clones. On the other hand Guzakirmizi *et al.*, (1993) observed significant differences in the strength and pattern of RAPDs from different regenerants of barley. Vallés *et al.* (1993) observed pre-existing polymorphism, but

failed to detect newly generated variation in the protoplast derived plants in meadow fescue.

In the present study about 15 obvious polymorphic DNA fragments were observed using different primers for amplification. But as compared with the total DNA fragments studied, the fraction of the polymorphic DNA fragments was very small and was found in only few of the samples studied.

In *Brassica* species no report was found about the use of RAPDs for the detection of somaclonal variation, therefore direct comparison of RAPDs results obtained here, with those of other workers was not possible. However, many workers have reported about the existence of somaclonal variation detected using some other techniques. Yamashita *et al.*, (1991) regenerated plants from mesophyll protoplasts of Brussel sprouts and observed morphological variations in number of stalks. Jain (1991) compared protoclonal variation with chemical mutagenesis in *Brassica napus* L. and reported that mutagenesis did not induce useful variation but protoclonal variation appeared exploitable for crop improvement. Xiong *et al.*, (1988) also reported protoclonal variation in cabbage. On the other hand Wamling (1993) regenerated plants from protoplasts of spring swede. Progenies of these regenerated plants tested over two years revealed no variation induced by the tissue culture, was established. Variation between the progeny lines was not greater than that within the varieties. Results of the present studies and reports of other researchers indicate that although, tissue culture regimes induce variation, it could not be demonstrated universally. Indeed there are still many reports not supporting the existence of somaclonal variation, either this is due to a true lack of variation or a failure of technique available for determining variation.

RAPD assay of different regenerated plants from the same callus piece, showed the chimeral nature of the callus at the point of regeneration. Plants derived from regenerated multiple shoots from a single callus piece showed DNA polymorphism for few of the DNA fragments. These differences in regenerated plants from the same callus piece provide evidence that the callus piece consisted of at least two types of genetically different cells. Possibly the callus piece might have a sectorial chimera and the plants regenerated were from different sectors and thus showed polymorphism. These results were not un-expected. The presence of chimeras in callus cultures was expected and has already indicated in many reports (Strause *et al.*, 1980; Hussey, 1986a; Hussey *et al.*, 1986b; Button and Kuchba, 1989). A mutational event in one

of the cells in a callus leads to chimerical callus. And further multiplication of mutated cell and regeneration of plants from these regions develop into variants. Whether or not these could be distinguished as variants, depends on the availability of appropriate technique. Some times chimera plants are developed from the chimera tissues which are of great importance especially in horticultural plants.

RAPD analysis of DNA from selected (for NaCl) and un-selected cultured material showed some variation in both types of samples. The variation observed in the two types of material was not specific. The amount of variation revealed by different samples to the parent plant was random. No obvious DNA polymorphism was observed which could be related to selected or un-selected material. These results coincide with the results of the stability test (Section 4.4.2) in which no stable salt resistance was found in curly kale and it has been suggested that resistance shown by the selected callus might be due to physiological adaptation. In the present study no specific RAPD marker related to culture induced genetic variation for salt resistance was observed. This might be either due to the limitation of the sample size and/or the number and type of primers tested, or the non-existence of genetic variability specific to salt tolerance. Previously other techniques have been used for the detection of genetic variation for salt resistance. Some of these reports provide evidence of the existence of genetic changes for salt tolerance, expressed by the tissue cultures in *Brassica* species (Chopra *et al.*, 1990; Jain (S) *et al.*, 1991a; Kirti *et al.*, 1991). While in some other studies stable salt resistant somaclones were not observed (Bressan *et al.*, 1985; McCoy, 1987a; Chandler *et al.*, 1988b).

It has been reported by many workers that variation in tissue culture increases with the duration of the cultures. Barbier and Dulieu (1983), using a genetically marked explant source, have shown that while most genetic changes occur in the first few mitoses in culture, some genetic changes increase with the duration of culture. Lörz and Scowcroft (1983) also reported that doubling the duration of culture time the frequency of genetic changes increased from 1.4 to 6%. The possible reason for the increase in variation with the increase in culture time is that when the cells are exposed to culture condition for more time there are more chances of culture induced mutations occurring. Despite the above evidence, the current study could not support these findings. In the present study no obvious increase in polymorphism was observed with the increase of culture time. Increase or decrease in the variation was at random, regardless of the culture time.

RAPDs have some technical limitations which may result in inaccurate interpretation. Mostly it happens in case of point mutations. Primers can bind (with reduced efficiency) to targets containing one or two mismatches (Bachmann, 1994). There will be an imperfect primer attachment site. On the other hand due to mismatches the point mutation(s), which are in the target region, may not be detected (Fig 5.14, Step C). These defects can not be completely excluded and it is safer to disregard weak and unreliable bands (Bachmann, 1994). Although, by disregarding the weak bands incorrect results can be avoided and reliability of the assay is increased, sometimes some additional information may be lost which were not due to mismatches. This must be a sacrifice of polymorphism as revealed by a particular primer in favour of robust results. Another limitation in RAPD analysis is that RAPD-PCR products are separated on the basis of segment length rather than segment sequence. Therefore changes in few bases, which are not present in the target regions, may not be detected (Fig 5.14, Step D). From the above discussion it can be suggested that the results obtained by RAPDs may have missed some useful information and weak bands observed in the profiles may be due to incorrect matching of the primer. Therefore it is beneficial to use as many primers as possible, to reduce the these defects.

### ***Conclusions***

It has been established by a number of workers that RAPD analysis has potential in the establishment and estimation of somaclonal variation (Vallés *et al.* 1993; Guzikirmizi *et al.*, 1993). The present study generally support this view. Whilst recognising that the technique does have limitations with regard to the estimation of point mutations.

Many authors (Larkin and Scowcroft, 1981; Robertson *et al.*, 1984; Jones, 1988; Jain *et al.*, 1993; Yamashita *et al.*, 1991; Jain, 1991; Jain *et al.*, 1991) have given substantial acknowledgement to the existence of variability among plants regenerated from *in vitro*. Therefore the observation of general variation arising via callus culture is supported by the results in this study.

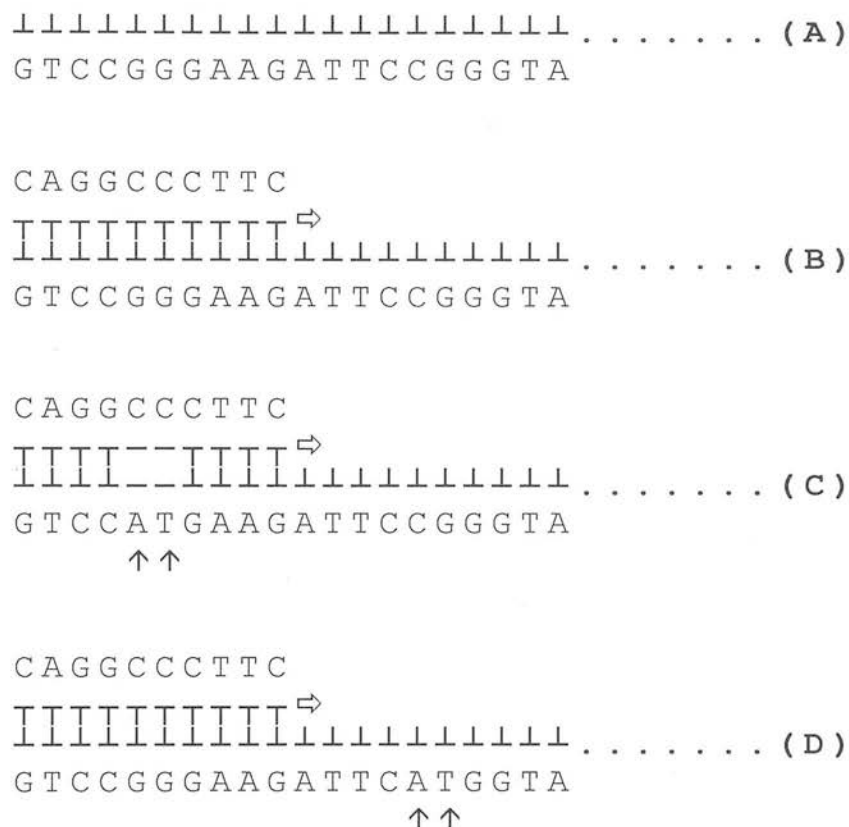


Fig. 5.14. Diagrammatic description of limitations of RAPDs, due to which point mutations may not be identified. A: is original template DNA, B: normal amplification using decamer primer, C: change in the template DNA in the target region and imperfect attachment, and D: change in the template DNA outside the target region. Amplification product in all the cases may be same.  $\uparrow$  indicates changed base.



The existence of somaclonal variation mainly depends on many factors, one may be chance mutation. Somaclonal variation may exist in some species or studies and may be absent from others. Therefore, although there is much support for the existence of variation in tissue cultures, there are still reports showing non-existence of somaclonal variation (Vamling, 1993). Whether that is true situation or not, may in practice reflect more on the technique available for the assessment of variation. Indeed many studies are involved with the identification of variants with specific traits. Further to identify such variants does not indicate that variation does not exist, only that variation for that specific trait was not been identified.

## 6. GENERAL DISCUSSIONS AND POSSIBILITIES FOR FUTURE RESEARCH

The general aim of this project was to investigate the effectiveness of *in vitro* selection for crop improvement. This would involve a study of callus culture in *Brassica* species and its requirements, and the identification and estimation of somaclonal variation in *Brassica* species in general, as well as specifically related to salt tolerance. However, partly due to limitations of time available and partly due to limitations of the techniques adopted, it was not possible to satisfy all aims. Nevertheless, the study has attempted to answer some relevant questions. In general *Brassica* species were found suitable for callus induction and subsequent callus cultures. Problems of regeneration were found in some of the species, which then could not be evaluated fully. *In vitro* callus selection was found to be an easy technique in its use, but the production of stable salt tolerant cell lines was questioned in some of the species under study. This may be due to some limitations in the selection technique used, but due the limitation of the time available, other techniques could not be fully evaluated for comparison. RAPD analysis was adopted in order to detect and estimate the level of somaclonal variation. The analysis provided some evidence for the existence of genetic variation in cultured lines. Specific genetic variation related directly to salt tolerance could not be examined due to possible limitations in the technique used and/or small sample size.

The initial aim of this study was to evaluate the potential of different *Brassica* species and different explant sources for *in vitro* culture and the requirements for different culture phases. In general the performance of *Brassica* species in tissue culture indicted that the genus has great potential for use as research tools in a tissue culture programmes. The results clearly indicate that callus induction and subsequent callus culture was achieved successfully from all the explant sources and species studied, although the callus growth differed with the explant source and the species. Plant regeneration was not equally successful from the different explant sources and species. Only one, out of three species studied showed successful plant regeneration. Although, plant tissue culture techniques have been used successfully for many years, regeneration from callus or cell culture is still problem for some crop species. According to George (1993) morphogenesis has now been observed *in vitro* in numerous plants of many genera, but it can not yet be induced universally. Even with in a species, varieties can be found which are recalcitrant.

Plant regeneration in some crop species may be difficult, but may or may not be impossible. It is evident from the literature that most plant species have potential for *in vitro* regeneration, which needs to be evaluated. In this project, the failure to obtain regeneration might be due to some limitations in the technique adopted and/or culture conditions. There are a number of factors which may have enhanced regeneration. Some of these may be related to stress as stimulant of regeneration (heat, salt, plant pathogens, electrical stimulation, gamma irradiation, growth inhibitors etc.), while the other appears to operate by entirely different mechanisms such as: calcium, cobalt, vitamin C, antibiotics, phenolics, amino acids, carbohydrates, glycoprotein, plant hormones (dicamba, abscisic acid and abscisic acid analogues, GA, BAP, 2,4-D etc.), activated charcoal, gelling agents and solidity of medium and low pH of medium (Herman, 1992). In this project only a few of the plant hormones, with a range of concentrations, were studied in any detail for plant regeneration. Obviously this range of potential variables with respect to regeneration, would require substantial experimentation, far beyond the time scale available for action.

Although plant regeneration is a limiting factor for the efficient use of *in vitro* selection in many crop species. *In vitro* selection may still have potential for crop improvement even if regeneration is not possible from the selected cells. Genetically stable salt resistant cell lines could also be used as donor (genetic) material (Evans *et al.*, 1984; Evans, 1986) which can be utilised by using advanced biotechnology such as protoplast fusion or gene transfer through recombinant DNA methodology (Hasegawa *et al.*, 1986), to develop new varieties.

The subsequent aim of this project was to examine the feasibility of *in vitro* selection and its usefulness in crop improvement. Use of callus culture, for *in vitro* selection in *Brassica* species, was found very easy, but the effectiveness of the use of callus culture for *in vitro* selection was questionable. *In vitro* selection is considered effective, if the resistance persists up to regenerated plants, and into the next generation. If regeneration is not possible, at least selected cells should show significantly more tolerance as compared with un-selected callus, even after growing on salt-free medium for a few generations. If this could be supported by additional information as revealed by a genetic marker to the character under study, then *in vitro* selection could be considered effective. In this project only one, out of three *Brassica* species, showed stable salt tolerance in tissue culture. The other two species

did not show stable salt tolerance. In these species although selected callus showed more salt tolerance as compared with the un-selected callus, the selected callus did not show significantly more salt tolerance after exposure to salt free medium for a few generations. In *Brassica rapa*, although selected and maintained (on salt free medium) callus showed significantly more salt tolerance as compared with the control callus, the tolerance was reduced and was less than that from callus which had not been exposed to the salt free medium after selection. These results indicated that although tolerance was increased in the selected callus, some of the 'enhanced' tolerance was lost when selected callus was exposed to salt-free medium. This loss, in 'enhanced' salt tolerance (which varied between species), may be due to limitations in the technique adopted. The most readily identifiable weakness in *in vitro* callus selection is that not all cells in a callus piece are directly in contact with the selective agent. The cells which were away from the medium may not be actually salt tolerant, but survived due to crossfeeding through other cells. There are other techniques, which may have potential to overcome these limitations. Among these techniques, the most commonly used are selection from fine suspension culture and selection from protoplast culture. Again due to limitation of time, in this project these techniques could not be fully evaluated in order to make a comparison between such techniques.

The other possible cause of the reversible 'enhanced' salt tolerance might be physiological adaptation of the cells to the stress. Most of the cells selected, showing salt tolerance may not be genetically changed, only physiologically adapted to the stress condition. When these cells were cultured on the salt free medium they lost their ability to grow under excess salt conditions. More physiological adaptations, with less genetic changes in tissue culture reduces the effectiveness of *in vitro* selection for stable resistance. Physiological adaptation of cells to excess salt concentrations has already been observed by many researches.

In order to evaluate the potential of *in vitro* selection it would advantageous to know in advance, the genetic nature of tolerant lines. Salt tolerance has been suggested as both a simple and complex character, by different workers. Similarly our present knowledge on the genetics of resistance to a range of abiotic stresses is less than perfect. Evaluation of *in vitro* selection might be more appropriate when knowledge of genetic nature is understood and a range of abiotic stresses of increasing levels of genetic complexity can be examined and compared in order to determine the type of genetic change which *in vitro* selection could used with effect. However, as with many other characters, this would not necessarily stop practising

plant breeding using the technique empirically in order to obtain resistance to an abiotic stress, but it would be used to gauge the likelihood of success.

The RAPD-PCR technique was adopted to satisfy the aim of detection and estimation of somaclonal variation in different cultured lines and regenerated plants. The technique was found easy in its use and has some potential for detection and estimation of somaclonal variation. The technique was found especially useful for a general overall estimation of variation. However, as with many techniques, there are limitations with RAPDs particularly in relation to the determination of point mutations and genetic changes associated with a specific character. There are many other techniques available which may have potential to over-come some of limitations of RAPD technique. These technique include RFLP, AFLP, gene sequencing, PCR for the amplification of a specific DNA fragment, use of clonal DNA libraries. In turn, these technique also may have some limitations in their use e.g. time required, hazardous chemicals, specificity in their use. Further more some of these techniques also require specific knowledge of the genetic control of the character under study. For characters of complex nature (thought by some workers to include salt tolerance), which may be controlled by more than one gene, or controlled by gene(s) with strong interaction with environment, or characters for which the complete genetic information is not known, these approaches may have less potential. Therefore for these characters study of the regenerated plants and their progenies, whenever possible, might be a better approach.

In this study somaclonal variation as estimated using RAPDs analysis, showed up to 11% variation as compared with the parent plants. Variation estimated by the RAPDs technique are based on the total genomic DNA content. In any organism the total DNA span is not active all the time, and at any time only a fraction of the total DNA is being expressed. Indeed in human beings only about 3% of total DNA contains genes, the type of DNA that codes for protein (Cohen, 1995). Furthermore all the genes may not be active all the times. Therefore total genetic variation, detected by the RAPDs may not being expressed at the time of analysis. Consequently actual expressible genetic variation may be just a fraction of the total variation detected by the RAPDs. On the basis of these findings, it can be suggested that the frequency of the variation expressed by the tissue culture may not be enough for crop improvement, through *in vitro* selection, in some crop species and for some traits. Although, nearly all the authors have recognised the potential value of somaclonal variation as a tool in plant breeding, there are still few exceptions in which

somaclonal variation has not been utilised due to unpredictability in some crop species (e.g. Challeg and Ray, 1984). However, a more direct approach to mutation/selection has been suggested by Beversdorf and Kott (1987), in which *in vitro* selection was performed after mutagenesis. Mutagenesis along with the tissue culture selection is used to increase variation within the cell population. Mutagenesis followed by *in vitro* selection has been successfully used by many researchers (e.g. Ahmad *et al.*, 1991; Swanson *et al.*, 1988). Therefore for the crop species or the character under study, if somaclonal variation is unpredictable, mutation induction along with *in vitro* selection may increase the potential of *in vitro* selection for crop improvement.

In general, it can be suggested that tissue culture technology has potential for crop improvement. The technique requires less time and less, but skilled labour. Effectiveness of *in vitro* selection, especially for salt tolerance, depends on the genetic changes expressed by the tissue culture, specific to the character under study and genotype in use. The potential of *in vitro* selection for crop improvement, may also vary with the technique adopted and abiotic stress under study. RAPDs analysis has great potential for over all detection and estimation of somaclonal variation. This approach may not be as reliable for the determination of point mutations and genetic changes specific to a character. Finally it is concluded that *in vitro* selection has considerable potential in plant breeding. However, due to limitations in the technique it is unlikely to be used alone and will join the other support technologies for future crop improvement.



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## APPENDICES

### Appendix 2.1.

#### Addresses of the suppliers

<b>Bassaire</b>	Bassaire, Duncan Road, Swanwick. Southampton, Hampshire.
<b>Crucifer Genetics</b>	
<b>Co-operative</b>	University of Wisconsin-Madison. Department of Plant Pathology. 1630 Lindon Drive, Madison, Wisconsin 53706, USA.
<b>Flowgen Instrument Ltd.</b>	Broad Oak Enterprise Village, Broad Oak Road, Sittingbourne, Kent, ME9 8AQ Ph: (01795) 429737 Fax: (01795) 471185
<b>Flow Laboratories</b>	Woodcock Hill, Harefield road, Rickmansworth, Herts, WD3 1PQ.
<b>Media™</b>	Phillip Harris Ltd. Lynn Lane Shentone, Lichfield, Staffordshire WS14 0EE.
<b>Nickerson</b>	Nickerson Seeds Ltd. Joseph Nickerson Research Centre. Rothwell, Lincoln LN7 6DT Official Seed Testing Station. Huntinton road, Cambridge CB3 0LE.
<b>Operon Technologies Ltd.</b>	1000 Atlantic Ave., Alameda, CA 94501, USA. Tel: (510) 865-8644 Fax (510) 865-5255
<b>Parafilm "M"®</b>	American National Can™. Greenwich, CT. 06836, USA.

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<b>Perkin-Elmer</b>	Perkin-Elmer Ltd. Beacon Fields, Bucks, HP9 1IQ. Ph: (01494) 676161
<b>Shamrock</b>	Irish Moss Peat, Bord na Mona, Dublin Levland.
<b>Sigma®</b>	Sigma-Aldrich Company Ltd. Fancy Road, Dorset, BH17 7NH, UK. St. Louis MO 63178, USA.
<b>Sterlin</b>	Biby-Sterlin Ltd. Stone, Staffordshire, ST15 0SA.

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**Appendix 2.2.****DNA isolation Kit**

The DNA isolation kit used in this project was supplied by the Flowgen Inst. Ltd. The supplier did not provide complete recipe of the kit, even after repeated request, due to the need to maintain company confidentiality. The information available about the kit are as.

<b>Name:-</b>	DNA isolation kit
<b>Product No.</b>	D5500A
<b>Manufacturer:-</b>	GENTRA System, Inc. 3905 Annapolls Lane, Minneapolis, MN 55447
<b>Supplier:-</b>	Flown Instrument Ltd Broad Oak Enterprise Village, Broad Oak Road, Sittingbourne, Kent, ME9 8AQ Ph: (01795) 429737 Fax: (01795) 471185

**Composition:-**

## Cell Lysis Solution:

EDTA, disodium salt  
Tris(hydroxymethyle) aminomethane. Tris Buffer  
SDS, sodium dodecyl sulfate

## DNA hydration solution:

EDTA; Tris buffer

## RNase solution:

Glycerol; Tris buffer



### Appendix 3.1

#### Establishment of fine suspension cultures and cell plating

A non-differentiating, friable callus line derived from the hypocotyl explants of different *Brassica* species and maintained by monthly subcultures, was used for the establishment of suspension cultures. Liquid culture medium was prepared (as described under Section 2.3, without phytogel), specific to the species, in 250 ml conical flasks with 100 ml medium in each. Suspension cultures were initiated by placing 10 friable callus pieces (approximately 100 mg each) in to the liquid medium. Flasks were sealed with sterile aluminium foil and then were placed on a gyratory shaker at 100 rpm in the culture room. A week later, the suspensions filtered through a double layer of sterile cheese cloth to remove the remaining pieces of callus and large cell aggregates. Suspensions were incubated for further on week, with continuous shaking and then the number of cells per ml medium were estimated using a Hamocytometer (Sigma). Cultures showing fine suspension with min 30000 cells ml<sup>-1</sup> were subcultured with 1:2 dilution (minimum inoculum density of 10000 to 15000 cells/ml; George and Shrington, 1984). Suspensions were maintained on gyratory shaker for 3 weeks and again filtered through a double layer of cheese cloth. Cells number in the liquid medium were again calculated. But the cultures could not reach the minimum inoculum density limit for subcultures and it appeared that cells were not multiplying actively in the liquid medium. The procedure was repeated several times with alterations to culture period and dilution rate, but no satisfactory results could be achieved.

Suspension cultures were also used for cell plating on solid culture medium. Solid culture medium was prepared and dispensed into petri dishes of 50 mm diameter. 2x (double strength) medium was also prepared separately, which was kept warm in an oven at about 45°C. Suspensions were mixed with the 2x culture medium (with 1:1 ratio) and quickly poured in a thin layer on the surface of solid medium in a petri dish. Petri dishes with plated cells were allowed to incubate in the culture room. No colonies developed from the single cells even 3 month after plating. This was repeated but no colony development was achieved.

Possible reason for this failure to establish viable cell growth in liquid medium include general culture media required, the viability of cells, the carbon source,

inoculum density. Each of these variable would require investigation in order to elucidate optimal requirement which would require substantial time.

**Appendix 3.1. Analysis of variation of callus fresh weight from hypocotyl explants of curly kale on different levels of NAA and BAP (a). Levels of growth regulators tested, ranged from 0-10 mg l<sup>-1</sup>, n=5.**

Source of variation	d.f	Sum of squares	Mean squares	F-ratio
NAA	3	15.181	5.060	102.84 **
BAP	3	4.882	1.626	32.829 **
Interaction	9	7.519	0.835	16.854 **
Residual	64	3.172	0.05	
Total	79	30.754		

**Appendix 3.2. Analysis of variation of callus fresh weight from hypocotyl explants of curly kale on different levels of NAA and BAP in combination (b). Levels of growth regulators tested, ranged from 0.5-5 mg l<sup>-1</sup>. n=5.**

Source of variation	d.f	Sum of squares	Mean squares	F-ratio
Treatments	9	10.992	1.221	4.719 **
Residual	40	10.352	0.259	
Total	49	21.343		

**Appendix 3.3. Analysis of variation of relative increase in callus fresh weight from Primary callus of curly kale on different levels of NAA and BAP, n=5.**

Source of variation	d.f	Sum of squares	Mean squares	F-ratio
Treatments	9	40.936	4.548	5.997 **
Residual	40	30.339	0.758	
Total	49	71.275		

**Appendix 3.4. Analysis of variation of callus fresh weight from hypocotyl explants of winter oilseed rape on different levels of NAA and BAP**  
**(a). Levels of growth regulators tested, ranged from 0-10 mg l<sup>-1</sup>. n=5.**

Source of variation	d.f	Sum of squares	Mean squares	F-ratio
NAA	3	20.286	6.762	92.477 **
BAP	3	15.75	5.25	71.799 **
Interaction	9	7.408	0.823	11.256 **
Residual	64	4.68	0.073	
Total	79	48.123		

**Appendix 3.5. Analysis of variation of callus fresh weight from hypocotyl explants of winter oilseed rape on different levels of NAA and BAP**  
**(b). Levels of growth regulators tested ranged from 1-10 mg l<sup>-1</sup>. n=5.**

Source of variation	d.f	Sum of squares	Mean squares	F-ratio
Treatments	9	14.183	1.576	6.348 **
Residual	40	9.93	0.248	
Total	49	24.114		

**Appendix 3.6. Analysis of variation of relative increase in callus fresh weight from Primary callus of Winter oilseed rape on different levels of NAA and BAP, n=5.**

Source of variation	d.f	Sum of squares	Mean squares	F-ratio
Treatments	9	70.903	7.878	5.674 **
Residual	40	55.533	1.388	
Total	49	126.437		

**Appendix 3.7. Analysis of variation of callus fresh weight from hypocotyl explants of *Brassica rapa* on different levels of NAA and BAP (a).**

Levels of growth regulators tested ranged from 0-10 mg l<sup>-1</sup>, n=5.

Source of variation	d.f	Sum of squares	Mean squares	F-ratio
NAA	3	1.502	0.501	15.713 **
BAP	3	2.408	0.803	25.184 **
Interaction	9	4.417	0.491	15.4 **
Residual	64	2.04	0.032	
Total	79	10.367		

**Appendix 3.8. Analysis of variation of callus fresh weight from hypocotyl explants of *Brassica rapa* on different levels of NAA and BAP (b).**

Levels of growth regulators tested ranged, from 0.5 -5 mg l<sup>-1</sup>. n=5.

Source of variation	d.f	Sum of squares	Mean squares	F-ratio
Treatments	9	4.919	0.547	5.463 **
Residual	40	4.002	0.1	
Total	49	8.921		

**Appendix 3.9. Analysis of variation for relative increase in callus fresh weight from Primary callus of *Brassica rapa*, n=5.**

Source of variation	d.f	Sum of squares	Mean squares	F-ratio
Treatments	9	107.899	11.989	7.749 **
Residual	40	61.882	1.547	
Total	49	169.781		

**Appendix 4.1. Analysis of variation of relative fresh weight gain from primary callus of curly kale, cultured on different levels of NaCl. n=5.**

Source of variation	d.f	Sum of squares	Mean squares	F-ratio
Treatments	7	84.145	12.021	15.509 **
Residual	32	24.802	0.775	
Total	39			

LSD=1.134,  $P=0.05$

**Appendix 4.2. Analysis of variation of relative weight gain from primary callus of winter oilseed, cultured on different levels of NaCl. n=5.**

Source of variation	d.f	Sum of squares	Mean squares	F-ratio
Treatments	7	54.599	7.8	49.382
Residual	32	5.054	0.158	
Total	39	59.654		

LSD=0.512,  $P=0.05$

**Appendix 4.3. Analysis of variation of relative weight gain from primary callus of *Brassica rapa*, cultured on different levels of NaCl. n=5.**

Source of variation	d.f	Sum of squares	Mean squares	F-ratio
Treatments	7	133.695	19.099	16.139
Residual	32	37.869	1.1183	
Total	39	171.563		

LSD=1.402,  $P=0.05$

**Appendix 4.4. Analysis of variance of callus fresh weight from hypocotyl explants of curly kale. Hypocotyls were cultured on MS-media supplemented with different levels of NaCl. n=5.**

Source of variation	d.f	Sum of squares	Mean squares	F-ratio
Treatments	7	29.531	4.219	77.916 **
Residual	32	1.732	0.054	
Total	39	31.263		

LSD=0.344,  $P=0.05$

**Appendix 4.5. Analysis of variance of callus fresh weight from hypocotyl explants of winter oilseed rape. Hypocotyls were cultured on MS-media supplemented with different levels of NaCl. n=5.**

Source of variation	d.f	Sum of squares	Mean squares	F-ratio
Treatments	7	35.054	5.008	53.937
Residual	32	2.971	0.093	
Total	39	38.025		

LSD=0.393,  $P=0.05$

**Appendix 4.6. Analysis of variance of callus fresh weight from hypocotyl explants of *Brassica rapa*. Hypocotyls were cultured on MS-media supplemented with different levels of NaCl. n=5.**

Source of variation	d.f	Sum of squares	Mean squares	F-ratio
Treatments	7	8.314	1.188	14.603
Residual	32	2.603	0.081	
Total	39	10.917		

LSD=0.367,  $P=0.05$

**Appendix 4.7. Analysis of variance of seedling fresh weight from different species of *Brassica*. Seed were germinated on different NaCl concentration. n=5.**

Source of variation	d.f	Sum of squares	Mean squares	F-ratio
Species	2	0.037	0.018	30.796 **
Salt Concentrations	7	0.659	0.094	158.805 **
Interaction	14	0.066	0.005	7.969 **
Residual	96	0.057	0.0006	
Total	119	0.819		

**Appendix 4.8. Analysis of variation of seedling fresh weight of curly kale, grown on different levels of NaCl. n=5.**

Source of variation	d.f	Sum of squares	Mean squares	F-ratio
Treatments	7	0.215	0.031	54.053 **
Residual	32	0.018	0.0006	
Total	39	0.233		

LSD=0.031,  $P=0.05$



**Appendix 4.9. Analysis of variation of seedling fresh weight of winter oilseed rape, grown on different levels of NaCl. n=5.**

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Treatments	7	0.392	0.056	171.443 **
Residual	32	0.010	0.0003	
Total	39			

**Appendix 4.10. Analysis of variation of seedling fresh weight of *Brassica rapa*, grown on different levels of NaCl. n=5.**

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Treatments	7	0.119	0.017	19.197 **
Residual	32	0.028	0.0009	
Total	39	0.147		

LSD=0.038,  $P=0.05$

**Appendix 4.11. Analysis of variation of relative increase in callus fresh weight from selected and un-selected, C8 callus of curly kale, cultured on different levels of NaCl, n=5.**

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Callus type	2	2.655	1.327	1.299
Media type	1	6.866	6.866	6.72 *
Interaction	2	0.623	0.311	0.305
Residual	24	24.518	1.021	
Total	29	34.661		

**Appendix 4.11 Analysis of variation of relative increase in callus fresh weight from selected and un-selected, C13 callus of curly kale, cultured on different levels of NaCl, n=5.**

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Callus type	2	1.008	0.504	0.636
Media type	1	11.694	11.694	14.773 **
Interaction	2	0.649	0.325	0.41
Residual	24	18.998	0.792	
Total	29			

**Appendix 4.13. Analysis of variation of callus fresh weight from leaf discs of regenerants, cultured on different levels of NaCl. Rgenerants were derived from selected and control callus, n=5.**

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Plants type	1	0.006	0.006	0.002
Media type	1	42.550	42.55	149.204 *
Interaction	1	7.938	7.938	0.003
Residual	16	4.563		
Total	19	47.115		

**Appendix 4.14. Analysis of variation of relative fresh weight gain from primary callus produced derived from leaf discs of regenerants, cultured on different levels of NaCl. Rgenerants were derived from selected and control callus, n=5.**

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Callus type	1	0.034	0.034	0.062
Media type	1	29.33	29.33	52.712 *
Interaction	1	1.3	1.3	2.338
Residual	16	8.903	0.556	
Total	19	39.567		

**Appendix 4.15. Analysis of variation of relative increase in callus fresh weight from selected and un-selected, C8 callus of winter oilseed rape, cultured on different levels of, n=5.**

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Callus type	2	1.143	0.572	0.273
Media type	1	55.592	55.592	26.595 **
Interaction	2	1.110	0.555	0.265
Residual	24	50.167	0.090	
Total	29			

**Appendix 4.16. Analysis of variation of relative increase in callus fresh weight from selected and un-selected, C13 callus of winter oilseed rape, cultured on different levels of, n=5.**

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Callus type	2	0.731	0.365	1.841
Media type	1	15.907	15.907	80.171 **
Interaction	2	0.852	0.426	2.146
Residual	24	4.762	0.198	
Total	29	22.251		

**Appendix 4.17. Analysis of variation of relative increase in callus fresh weight from selected and un-selected, C9 callus of *Brassica rapa*, cultured on different levels of, n=5.**

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Callus type	1	5.333	5.333	4.092
Media type	1	59.205	59.205	44.73 **
Interaction	1	8.917	8.917	6.737 *
Residual	16	21.177	1.323	
Total	19	94.633		

**Appendix 4.18. Analysis of variation of relative increase in callus fresh weight from selected and un-selected, C9 callus of *Brassica rapa*, cultured on salt free medium, n=5.**

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Treatment	1	0.229	0.229	0.106
Residual	8	17.217	2.152	
Total	9	17.446		

LSD=  $P=0.05$

**Appendix 4.19 Analysis of variation of relative increase in callus fresh weight from selected and un-selected, C9 callus of *Brassica rapa*, cultured on MS-medium with 1% NaCl, n=5.**

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Treatment	1	14.021	14.021	28.322 **
Residual	8	0.495	0.495	
Total	9	17.91		

**Appendix 4.20. Analysis of variation of relative increase in callus fresh weight from selected and un-selected, C13 callus of *Brassica rapa*, cultured on different levels of, n=5.**

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Callus type	2	30.946	15.473	5.574 *
Media type	1	48.646	48.646	17.524 **
Interaction	2	3.996	1.998	0.720
Residual	24	66.624	2.776	
Total	29	150.212		

**Appendix 4.21. Analysis of variation of relative increase in callus fresh weight from selected and un-selected, C15 callus of *Brassica rapa*, cultured on different levels of, n=5.**

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Callus type	1	5.234	5.234	3.296
Media type	1	29.669	29.669	18.682 **
Interaction	1	18.696	18.696	11.772 **
Residual	16	25.410	1.588	
Total	19	79.009		

**Appendix 4.22. Analysis of variation of relative increase in callus fresh weight from selected and un-selected, C15 callus of *Brassica rapa*, cultured on salt free medium, n=5.**

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Treatment	1	2.073	2.073	0.671
Residual	8	24.712	3.089	
Total	9	26.784		

**Appendix 4.23. Analysis of variation of relative increase in callus fresh weight from selected and un-selected, C15 callus of *Brassica rapa*, cultured on MS-medium with 1% NaCl, n=5.**

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Treatment	1	21.85	21.858	250.455 **
Residual	8	0.698	0.087	
Total	9	22.556		

**Appendix 4.24 Analysis of variation of relative increase in callus fresh weight from selected and un-selected, C16 callus of *Brassica rapa*, cultured on different levels of, n=5.**

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Callus type	3	7.670	2.557	1.411
Media type	1	75.129	75.129	41.471 **
Interaction	2	19.023	6.341	3.5 *
Residual	32	57.971	1.812	
Total	39	159.794		

**Appendix 4.25. Analysis of variation of relative increase in callus fresh weight from selected and un-selected, C16 callus of *Brassica rapa*, cultured on salt free medium with, n=5.**

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Treatment	1	2.287	0.672	0.267
Residual	8	45.663	2.854	
Total	9	47.949		

**Appendix 4.26. Analysis of variation of relative increase in callus fresh weight from selected and un-selected, C16 callus of *Brassica rapa*, cultured on MS-medium with 1% NaCl, n=5.**

Source of variation	d.f.	Sum of squares	Mean squares	F-ratio
Treatment	1	24.407	8.136	10.575 **
Residual	8	12.309	0.769	
Total	9	36.716		

LSD=1.176,  $P=0.05$

(Note:- '\*' Significant at  $P>0.05$

'\*\*' Significant at  $P>0.01$ )

**“Cheer up the worst is yet to come”**

(P.C. Johnson, 1920)